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EFFECTS OF CALCIUM ADMINISTERED PARENTERALLY TO THE NORMAL AND THE PARATHYROIDECTOMIZED DOG

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Clinicians as a rule have established definite safeguards for the intravenous injection of calcium. These precautions have arisen from the demonstrated toxicity of calcium ions in animal experiments (1), the various electrocardiographic changes in man (2), and the report of sudden death in two patients receiving digitalis immediately after the intravenous administration of calcium (3). Our interest in the problem arose from the necessity of treating patients suffering from post-operative tetany. It seemed important again to study experimentally the mechanism of death produced by calcium injections. Results will be reported from two types of experiments: (a) continuous injection of a solution of calcium gluconate until death, into dogs with normal and low blood calcium levels (parathyroidectomized) and (b) a single injection of a toxic dose of calcium salts.

A. CONTINUOUS INJECTION OF CALCIUM GLUCONATE UNTIL DEATH. *Experimental methods.* The dogs used in the experiments weighed 15 to 35 kgm. This allowed the withdrawal of samples without significant alterations of the blood volume. In the case of the parathyroidectomized animals, the experiments were performed 2 to 3 days after the operation. Nembutal anaesthesia (35 mgm. per kilogram) was used, a cannula was inserted in the carotid artery so that pulse and blood pressure tracings could be obtained, electrocardiograms (lead 2) were taken at intervals, and blood samples were drawn for analysis. Following the technique of Lieberman (4) an 8.4 per cent calcium gluconate in distilled water was injected at the rate of 4 cc. per minute into the left femoral vein until death (fall in the blood pressure to zero). The serum proteins were determined by the micro-Kjeldahl method, the calcium by the Clark-Collip modification of the Kramer-Tisdall procedure (5), and the phosphorus by the method of Youngburg (6).

RESULTS. *Stages of calcium action.* If one observes the blood pressure tracings taken from normal and parathyroidectomized dogs during the continuous injection of calcium, and correlates them with the blood chemical and electrocardiographic results, three stages may be distinguished. In the *first stage*, which lasted upwards of 5 minutes, a maximum of 20 cc. of calcium gluconate (1.68 grams) was injected. The blood pressure in this period remained normal (around 160 mm. Hg). At the end of this stage the heart

showed slowing and an increase in the amplitude as the second stage was initiated. The serum calcium values were increased up to a level of 25 mgm. In the *second stage*, which often lasted as long as $1\frac{1}{2}$ hours, the blood pressure was slightly elevated (10 to 30 mm.) at the early part and normal to lower (20 to 40 mm.) towards the end. The cardiac slowing noted at the beginning might be continued for a time but was eventually replaced by a tachycardia. The serum calcium levels now ranged between 25 and 75 mgm. per cent. The volume of fluid injected was proportional to the survival time of the animal and often amounted to 400 cc. Sudden death might occur at any time in this stage. If the dog survived, the *third stage* was initiated by a gradually falling blood pressure in which cardiac irregularities, slowing of the rate, and changes in the amplitude were noted. The calcium levels at this time were between 75 and 250 mgm. Some animals tolerated as much as 1000 cc. of fluid.

Electrocardiographic changes. These are based on a study of 13 normal animals. During the first stage no changes were noted. In the second stage there was a slight prolongation of the PR interval (range 0.04 to 0.08 second) and at times the P waves were absent. The QRS frequently showed lower voltages, slurrings and notchings. At times the S waves appeared when they had been previously absent. The T waves showed inversion, and at other times increased prominence. In nine animals sudden death occurred without a previous onset of changes other than those noted in the second stage. Four animals from which tracings were taken survived the second stage and had blood calcium levels in excess of 75 mgm. One of these died in cardiac arrest after a previous run of ventricular fibrillation. One died in ventricular fibrillation preceded by a slowing of the rate (90 to 30), lengthening of the PR interval (0.04 second) and an increase in the prominence of the T wave. A final one died with ventricular fibrillation preceded by a run of extrasystoles. These findings are quite similar to those reported by Smith, Hoff and Winkler (7).

Electrocardiographic changes reported by other workers in experimental animals consist of changes in rate, premature beats, shifts of the auricular pacemaker, varying degrees of block, alterations of the T wave, deepening of the S wave, ventricular fibrillation and death (8). In man the earliest corresponding changes are vagus-like actions, bradycardia, sinus arrhythmia, shifting of pacemaker, various degrees of heart block; the later actions—foci of idiopathic ventricular rhythm, ventricular extrasystoles, flattening or inversion of T waves, notching of QRS (2, 9)—are on the ventricle.

Changes in serum proteins. The serum proteins levels following parathyroidectomy were not altered significantly. With the intravenous injection of calcium in both the normal and the parathyroidectomized animals there was a gradual and progressive fall in the percentage of serum proteins. Although some of the animals survived for longer periods of time and received correspondingly larger volumes of calcium solution, they did not show significantly

lower protein values than animals which had smaller quantities of fluid. Thus eight animals (table 1) receiving injections of 500 cc. or more showed an average fall of serum proteins of 37 per cent, while six receiving less than 500 cc. showed an average fall of 40 per cent.

If one compares the fall in the serum proteins in the normal (39 per cent) and the parathyroidectomized animals (38 per cent) there is apparently no difference. The fall is not related to the initial blood calcium levels nor to the total quantity of calcium gluconate injected.

Inorganic phosphorus. In normal animals the inorganic phosphorus level rose definitely as the calcium levels increased (4 to 15 mgm.) but there was no

TABLE 1
Effects of continuous injection of calcium gluconate until death

	DOG NUMBER	TOTAL CALCIUM INJECTED*	SERUM CALCIUM LEVEL		SERUM PROTEIN LEVEL		PER CENT FALL
			Before	Terminal	Before	Terminal	
			mg. per 100 cc.	mg. per 100 cc.	grams per 100 cc.	grams per 100 cc.	
1. Normal dogs	B1	670	10.6	240.0	6.89	3.23	53
	B2	250	10.4	74.3	6.1	3.9	36
	B3	270	8.3	74.1	5.77	1.38	76
	B4	500	9.2	74.2	6.44	4.46	31
	B5	390	8.5	101.5	6.24	4.23	32
	B6	670	9.1	77.1	8.2	5.3	35
	B7	600	11.5	42.0	6.23	5.26	14
	B8	1,000	12.3	46.2	7.6	4.96	35
						<i>Average</i>	39
2. Parathyroidectomized dogs	C1	1,013	6.8	144.6	7.5	2.95	60
	C3	218	8.3	38.1	7.5	5.3	30
	C4	740	6.0	58.7	6.5	3.27	50
	C5	225	9.2	37.5	6.5	3.26	50
	C6	180	4.4	67.5	5.69	4.70	18
	C7	600	4.6	49.8	6.83	5.38	21
						<i>Average</i>	38

* Calcium gluconate, 8.4 per cent.

definite linear correlation. In one parathyroidectomized animal there was little change in the phosphorus level despite the marked increase in the calcium level.

Lethal factors. Reference to table 1 shows that there is no predictable level of blood calcium in either the normal or parathyroidectomized animals at which death will occur. In the 8 normals the average amount of calcium solution injected before death amounted to 540 cc. In the 6 parathyroidectomized animals with low initial blood calcium levels the corresponding figure was 496 cc. (8 per cent less than in normal animals). An initial low

level of calcium resulting from the parathyroidectomy did not protect the animal against calcium injections. It has been previously noted (10, 11, 4) that high concentrations of calcium reaching the heart cause immediate death. If the blood calcium is raised more slowly (4) much larger quantities may be tolerated before death ensues. Our experiments agree with those of others in that the cause of death is cardiac arrest following ventricular fibrillation and that in the course of the injections the imminence of death cannot be predicted. We believe that early deaths are attributable to a primary calcium effect on the heart. However, the tolerance of some animals for large quantities (1000 cc. and 1013 cc.) of calcium gluconate and the striking lowering of the serum proteins suggest that an additional site of calcium action should be sought.

Until the terminal stages the blood pressure was well maintained and the urinary secretion was continuing at a time when a fall in serum proteins had already been established. The effect of the constant injection of the calcium solution was to maintain the blood volume and prevent a fall in blood pressure. There was therefore no gross discrepancy between the blood volume and vascular bed, and it was obvious that our experimental objective now should be a study of the disappearance of the serum proteins. It was therefore decided to give a toxic dose of calcium in a single injection and to follow the alterations in plasma volume and serum proteins produced by this injection.

B. SINGLE INJECTION OF A TOXIC DOSE OF CALCIUM SALTS. *Experimental procedures.* A blood volume determination using the dye T 1824 (Evans blue)(12) was first done. This was followed immediately by an injection of a solution of 8.4 per cent calcium gluconate, amounting on the average to 17.7 cc. per kilogram of body weight. A second blood volume determination was done from 15 to 90 minutes after completion of the injection. In some of the experiments nembutal was used as an anaesthetic, in others no anaesthetic was used. For the plasma volume determinations the technique of Gibson and Evans (13) was employed with the substitution of a photo-electric colorimeter for the spectro-photometer. The dye was dissolved in 0.85 per cent sodium chloride to make a 0.5 per cent solution. It was then stored in ampules so that 11 to 12 cc. could be withdrawn. Ten cubic centimeters (50 mgm.) of the dye were injected into the animal and a remaining 1 cc. diluted to 200 cc. was used for the calibration curve. Since the average plasma volume of the dogs was 1000 cc., this made the dilution of dye within and without the animal comparable. For the calibration curve the dilutions were made by using a series of 5 tubes. Into each tube was placed 1 cc. of serum, diluted to 10 with saline solution, varying volumes of the dye (2.5 cc., 2.0 cc., 1.5 cc., 1.0 cc. and 0.0 cc.) and sufficient saline (0.85 per cent) to make up the final volume to 10 cc. The disappearance of the dye from the plasma was determined by withdrawal, from the dog, of six samples at 10 minute intervals. One cubic centimeter of serum from each of these samples was diluted to 10 for comparison with calibration curve. The second blood volume was estimated in a similar manner after a new calibration curve was made.

RESULTS: Control experiments. In table 2 are summarized the studies on normal dogs of successive blood volume determination. When the plasma

volumes were repeated on the same day, four hours were allowed to elapse between the first and second determinations. The agreement between the figures for plasma volume is quite satisfactory. In each case a third estimation of plasma volume was made 7 days later with similar agreement. Gibson and Evans (13) have reported that repeated sampling of blood from the vein causes a drop in the cell:plasma ratio. In this event there would of course be a corresponding fall in the cell and blood volumes. This may account for

TABLE 2
Repeated blood volume determinations on normal dogs

ANIMAL	DATE	PLASMA VOLUME	CHANGE IN PLASMA VOLUME	HEMATOCRIT	BLOOD VOLUME	CELL VOLUME	CHANGE IN CIRCULATING CELLS	CIRCULATING SERUM PROTEIN	CHANGE IN CIRCULATING SERUM PROTEIN
		cc.	per cent	per cent cells	cc.	cc.	per cent	grams	per cent
1	2/21	1,146		53.6	2,471	1,325		73.3	
		1,195	+4.3	53.4	2,566	1,371	+3.5	73.0	-0.4
	2/28	1,175	+2.5	49.9	2,346	1,171	-11.6	68.3	-7.3
2	3/3	1,295		43.1	2,279	983		82.6	
		1,290	-0.4	33.1	1,929	639	-35.0	79.7	-3.7
	3/10	1,308	+1.0	36.6	2,065	757	-23.0	85.6	+3.7
3	3/4	826		61.8	2,165	1,339		53.3	
		831	+0.6	58.3	1,993	1,162	-13.3	53.1	-0.47
	3/11	871	+5.5	57.0	2,027	1,156	-13.7	59.5	+11.6
Blood volumes before and after injection of 400 cc. 0.85 per cent sodium chloride									
4	1/6	1,121		39.9	1,866	744.3		73.5	
		1,218	+8.7	34.6	1,862	644.0	-13.5	72.7	-1.1
5	1/10	1,196		33.8	1,806	609.6		71.5	
		1,302	+8.9	34.0	1,972	669.5	+9.8	72.5	+1.4

the difference in some of our hematocrit readings (table 2), and emphasizes the fact that the dye method measures primarily plasma volume. Other methods are required to measure directly the cell volumes.

When the blood volume was increased by the intravenous injection of 400 cc. of isotonic salt solution there was a slight increase in plasma volume and a drop in the percentage of serum proteins, but there was no alteration in the total circulating serum proteins. The hematocrit values were variable. In a slightly different type of experiment, in which the control blood volume estimation was made one week prior to the experiment, similar results were

obtained. In these experiments the quantity of saline injected (800 to 1200 cc.) was equivalent to the animal's plasma volume. One hour was consumed in the injection of both calcium and salt solution and $1\frac{1}{2}$ hours were allowed to elapse before the second estimation of blood volume was made. If it had been determined somewhat earlier, the diluting effect of the salt solution on the plasma volume would doubtless have been more evident, but there still would have been no change in the total amount of circulating serum protein.

Blood volumes in unanesthetized dogs before and after the injection of calcium salts. The fall in plasma volume from 13.1 to 47.9 per cent (table 3) after the injection of calcium salts is strikingly significant. The corresponding reduc-

TABLE 3

Blood volumes of dogs before and after injection of calcium salts without anesthesia

DATE	TOTAL CALCIUM INJEC- TED*	PLASMA VOLUME	CHANGE IN PLASMA VOLUME	HEMATO- CRIT	BLOOD VOLUME	CELL VOLUME	CHANGE IN CIRCULATING CELLS	CIRCULATING SERUM PROTEIN	CHANGE IN CIRCULATING SERUM PROTEIN
	cc.	cc.	per cent	per cent cells	cc.	cc.	per cent	grams	per cent
1/ 9/40		891.3		56.9	2,068	1,177		53.3	
	400	426.6	-47.9	72.5	1,553	1,126	-4.3	31.8	-40.4
2/27/40		1,214		56.0	2,756	1,543		86.8	
	385	861	-29.1	67.3	2,633	1,772	+14.8	68.9	-20.7
2/14/40		755		48.2	1,477	722		44.3	
2/20/40	244†	473	-37.4	63.6	1,300	827	+14.5	34.4	-22.4
12/ 8/39		1,016		41.1	1,726	710		65.0	
12/27/39	300	883	-13.1	49.2	1,740	856	+20.6	49.7	-23.5

* 8.4 per cent calcium gluconate unless otherwise indicated.

† 2.76 per cent $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ instead of 8.4 per cent calcium gluconate.

tion in circulating serum proteins indicates that there has been a gross seepage of the plasma from the vascular bed. The rise in the hematocrit readings and the increase in the volume of circulating cells suggest that during the calcium injection fresh supplies of blood have been washed from the "blood depots." The augmented blood volume is subsequently reduced by the loss of plasma despite the retention of the cells. The vascular channels have become permeable to the plasma proteins but remain impermeable to the red blood cells.

In three experiments (table 4) there was a loss in serum proteins without significant change in plasma volume. When the toxic effects of calcium are less evident the animal may maintain its plasma volume by the addition of water and electrolytes during a period in which there is a progressive loss of serum protein. When the calcium action becomes more toxic this protective

mechanism breaks down and the water and protein alike escape through the vascular walls.

Blood volumes in dogs anesthetized with nembutal before and after the injection of calcium. Although the experiments detailed above appeared significant it seemed important to repeat them on anesthetized animals. Under these conditions reflex changes due to sampling and injection, also changes due to muscular activity and struggling (14, 15) would be eliminated and the technical details of the experiment would be simplified.

Control volumes under nembutal anesthesia. Hamlin and Gregersen (14) have reported a 10 per cent increase in plasma volume in cats anesthetized with nembutal. We could find no other studies of the effects of nembutal on blood volume. For this reason blood volume estimations were made before

TABLE 4

Plasma volume changes in dogs after injection of calcium gluconate without anesthesia

DATE	TOTAL CALCIUM INJECTED*	PLASMA VOLUME	CHANGE IN PLASMA VOLUME	HEMATO- CRIT	BLOOD VOLUME	CELL VOLUME	CHANGE IN CIRCULATING CELLS	CIRCULATING SERUM PROTEIN	CHANGE IN CIRCULATING SERUM PROTEIN
	cc.	cc	per cent	per cent cells	cc.	cc.	per cent	grams	per cent
1/18		632		56.0	1,438	805		44.1	
1/19	400	705	+11.5	50.5	1,426	721	-10.4	30.3	-31.4
3/5		1,075		51.6	2,222	1,147		64.0	
	340	1,018	-5.3	48.4	1,972	955	-16.7	56.7	-11.4
2/8		1,012		52.6	2,145	1,133		60.3	
	330	960	-5.1	54.1	2,089	1,129	-0.35	50.6	-16.2

* 8.4 per cent calcium gluconate.

and after nembutal anesthesia on four dogs, two experiments being made on each animal. There was no appreciable change in the plasma volume. The average change of all the experiments amounted to -2 per cent. There was, however, a fall in the hematocrit reading and a corresponding decrease in the total circulating cells varying between 16 and 44 per cent. Other workers (16, 17, 18, 19) have shown that, following the administration of sodium amytal, a maximal dilatation of the spleen occurs, associated with a fall in hemoglobin and a decrease in peripheral erythrocytes. Essex, Seeley, Higgins and Mann (18) noted that these changes in the erythrocyte count, hemoglobin concentration, and hematocrit reading following amytal anesthesia did not occur in splenectomized animals, and we have confirmed this in six experiments on three splenectomized dogs under nembutal anesthesia. These experiments confirm the results of other workers (19) as to changes in cell distribution and indicate that under nembutal as well as amytal there is a

sequestration of erythrocytes within the spleen. In normal dogs nembutal caused a drop of 8 per cent in total circulating serum proteins and 2 per cent in the plasma volume. In the splenectomized dogs nembutal caused no change in plasma volume and a decrease of only 3 per cent in total circulating serum proteins. These experiments would indicate that in the redistribution of the blood discussed above there may be slight changes in the serum proteins also.

Effects of calcium injections. The experiments showing the effects of calcium injections in dogs under nembutal (table 5) yielded results similar to those observed in unanesthetized dogs. It should be remembered that these

TABLE 5

Blood volumes of dogs before and after injection of calcium gluconate under nembutal anesthesia

DATE	TOTAL CALCIUM INJECTED*	PLASMA VOLUME	CHANGE IN PLASMA VOLUME	HEMATO- CRIT	BLOOD VOLUME	CELL VOLUME	CHANGE IN CIRCULATING CELLS	CIRCULATING SERUM PROTEIN	CHANGE IN CIRCULATING SERUM PROTEIN
	cc.	cc.	per cent	per cent cell	cc.	cc.	per cent	grams	per cent
12/30/40		1,244		44.4	2,237	993.2		77.0	
	460	1,039	-16.5	55.6	2,342	1,302.0	+31.6	63.5	-17.5
1/13/41		1,958		40.8	3,307	1,348		110.0	
	600	1,703	-13.0	51.4	3,510	1,805	+33.9	97.5	-11.3
2/ 3/41		1,099		54.0	2,390	1,291		68.1	
	680	981.4	-10.7	65.5	2,843	1,862	+44.2	62.6	-8.1
1/17/41		1,008		38.6	1,641	632.7		69.4	
	480	1,076	+6.8	40.5	1,808	732.1	+11.6	63.8	-8.0

* 8.4 per cent calcium gluconate.

animals had received nembutal before estimation of the first blood volume and that some slight decrease had doubtless occurred in the serum proteins, a larger decrease in the hematocrit readings and in circulating red cell volume. After the calcium injection the plasma volume and serum proteins were decreased further but the hematocrit reading and the total circulating red cell volume increased sharply. These additional cells represent blood added to the circulation from the "depots" in response to the effects of diminished blood volume. The magnitude of concentration of the blood is shown in the hematocrit changes in tables 3, 5 and 6. The progressive concentration in the blood is better seen in table 6. Here the hematocrit reading has increased almost 50 per cent and the percentage of serum proteins has risen 30 per cent. This would indicate that the water is being drained out of the vascular bed more rapidly than the proteins. It is interesting to note that Collip and Clark

(19) observed marked increases in the hemoglobin concentration of the blood in the terminal stages in animals who had received overdosages of parathyroid hormone. They suggested that the rise in hemoglobin concentration would indicate a great decrease in the plasma volume.

In another experiment the oxygen capacity of the venous blood rose from 21.7 per cent before calcium injection to 28.3 per cent, 171 minutes after the injection. During the same period the oxygen saturation fell from 73 to 41 per cent. Towards the end of the survival period increase in cardiac rate and Cheyne-Stokes respirations were noted. It is obvious that the loss of plasma and the increase of cells has increased the viscosity of the blood and slowed its rate of circulation. It has become a less efficient mechanism for the transport of oxygen. One can easily understand that an anoxic state would result and this might lead to ventricular fibrillation. Under these circumstances

TABLE 6
Progressive changes in blood following calcium injection

TIME	HEMATOCRIT	HEMO- GLOBIN	SERUM PROTEIN	SERUM CALCIUM
	per cent cells	grams per 100 cc.	grams per 100 cc.	mgm. per 100 cc.
Before Ca....	40.8	14.6	5.62	9.5
32 minutes after Ca	51.4	18.1	5.72	34.8
99 minutes after Ca.....	55.8	19.6	6.36	28.4
156 minutes after Ca.....	57.4	19.6	6.72	28.7
216 minutes after Ca.....	58.1	20.2	7.24	25.5
276 minutes after Ca.....	60.4	20.7	7.14	21.9
At death 406 minutes after Ca.....	60.2	20.9		

it would not be necessary to assume a primary calcium effect on the heart muscle.

Autopsy findings. Prior to death it was difficult to draw blood from the veins even with a large needle. At autopsy the cut tissues showed little or no tendency to bleed. Multiple hemorrhagic areas were noted beneath the endocardium, in the mucosa of stomach and intestine, under the peritoneal covering of small intestines, in the bladder, and on the surface of the pancreas. Areas of infarction were observed in the lung and spleen. Apparently in the terminal stages the separation of the capillary endothelial cells had become sufficient to allow an actual extravasation of blood cells with production of hemorrhagic areas.

CONCLUSIONS

1. When a solution of calcium gluconate was injected into normal and parathyroidectomized dogs until death, it was noted that death was not related to the quantity of fluid injected nor to the blood calcium level prior to injection.

tion or at the time of death. Electrocardiographic tracings showed either cardiac arrest or ventricular fibrillation. The serum proteins fell progressively. The fall amounted on the average to 40 per cent.

2. When a single toxic dose of calcium gluconate was injected intravenously into anesthetized (nembutal) and unanesthetized dogs, certain changes in the circulating fluids occurred. The order of the changes, which depend on the degree of the toxic action of the calcium, was as follows:

a. Serum proteins are lost but the plasma volume is maintained by the absorption of water and electrolytes.

b. Serum proteins and water are lost with a reduction in plasma volume; red blood cells are added to the circulation from "blood depots" and retained with a rising hematocrit figure, thus establishing an experimental polycythemia.

c. Finally, both plasma protein and cells escaped from the vascular channels giving rise to multiple small hemorrhagic areas in various viscera.

3. In control experiments under nembutal anesthesia there was no change in the plasma volume but there was a slight decrease in total circulating serum proteins and much larger decreases in the circulating red cell volume. The site of action of the nembutal is the spleen since these changes did not occur in splenectomized animals.

4. When concentrated calcium solutions are injected rapidly death may occur from the direct effect of the calcium on the heart muscle. When toxic doses of calcium are injected more slowly, it is suggested that death results from cardiac anoxia attributable to the viscous slow-moving polycythemic blood.

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THE DETERMINATION OF CHLORAL HYDRATE, CHLOROFORM, AND RELATED SUBSTANCES IN BLOOD¹

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Apparently all R-C-X,* compounds (1) give the Fugiwara color reaction (2) by which a pink to red color, depending on concentration, develops when they are heated with strong alkali and pyridine. Adaptation of this reaction to quantitative colorimetric methods is hampered by rapid fading of the color produced. Simultaneous preparation of standard and unknown solutions, and dependence on equal rates of fading of standards and unknowns, are employed in the Friedman and Calderone method (3) for chloral hydrate. In the determination of chloroform the standards for comparison are calibrated dye solutions in the method of Cole (4), and color plates in the method of Gettler and Blume (5). The difficulty and inconvenience of preparing accurate standard solutions simultaneously with unknowns are increased with chloral hydrate, chloroform, etc., because of their volatility and because their solutions do not keep well. On the other hand, the sacrifice of accuracy when comparing fading solutions with fixed colors is apparent. All methods provide for retardation of color fading by rapid cooling of solutions after color development.

The need in absorption studies for a rapid, convenient method of determining accurately small quantities of chloral compounds in blood led to the present investigation. A photoelectric colorimetric method based on the Fugiwara reaction was developed in which calibration curves replace standard solutions. Since all R-C-X₃ compounds produce the same color, the method is readily adapted to any one of these compounds by preparation of the appropriate calibration curve. The method was made practical and accurate by discovering a means of retarding color fading to a greater degree than in previous methods.

EXPERIMENTAL. Preliminary. The following series of experiments provide the basis for the technic of the method reported:

I. Colorimeter filter. The Rubicon Filter No. 540 was found to be satisfactory for reading the color produced by the Fugiwara reaction. It is more satisfactory

¹ A preliminary report of this investigation was presented before the American Society for Pharmacology and Experimental Therapeutics in Chicago, April, 1941.

* X = halogen.

than Filters Nos. 420, 440, 520, 580, 620, and 660 of the same series, and is standard equipment on Evelyn colorimeters (6).

II. Solution clarity. The cause of frequent failure to obtain solutions of optical clarity was sought. *Traces of stop-cock grease were found to cause the cloudiness. Coincident with this was the disclosure that certain stop-cock greases give a positive Fugiwara reaction, as shown by a pink to red color in blank determinations.* The method of Friedman and Calderone (3) provides for filtration of the solutions after color development, and before reading in the colorimeter, to obtain a clear solution. In the present method the color is developed directly in a colorimeter tube, and elimination of filtration is desirable both for convenience and for avoiding the fading which occurs during the time of filtration. Various stop-cock lubricants were tried before all were discarded in favor of a procedure requiring none.

III. Rate of color fading. The rate of fading was found to vary with the intensity of the color, and to decrease as the color faded. Also it was evident that the most accurate readings are made immediately after color development, which emphasized the increase in accuracy that further retardation of fading would produce. In addition, it was demonstrated that the error of methods using simultaneous preparation of standard solutions increases with the difference between the concentration of the unknown and the standard solutions.

IV. Retarding color fading. Among the unsuccessful attempts to retard color fading were: (1) independent variation of the concentration of each constituent of the reaction mixture, (2) variation of the degree and duration of heating the mixture, and (3) the use of ice-cold reagents to forestall slow reaction at room temperature. However, these observations were of value in developing the technic of the final method.

Successful retardation of fading resulted from the use of alcohol as diluent in the step following color production. Alcohol was found (1) to give greater clarity to the solution, (2) to insure miscibility of the pyridine, and (3) to retard color fading considerably more than water. Figure 1 shows a typical comparison of fading with water (curve 3) and alcohol (curve 2) as diluents. The latter rate was about 25 per cent of the former. Six cc. volumes of diluents were necessary for this comparison because a 5 cc. volume in the case of water results in immiscibility with pyridine. Curve 1 is a typical fading curve when 5 cc. of alcohol, as employed in the final method, is the diluent. This retardation of fading makes possible the accuracy obtained by the present method.

V. Accuracy. The method was tested by adding known amounts of chloral hydrate to whole blood or to blood filtrates, and, as shown in table 1, the error did not exceed 6.5 per cent even when the amount of chloral hydrate was extremely low.

VI. Colorimeter light intensity. In the absence of a blood blank determination the initial adjustment of the photoelectric colorimeter light intensity is made with a tube of distilled water, so that the intensity is at the point which the average

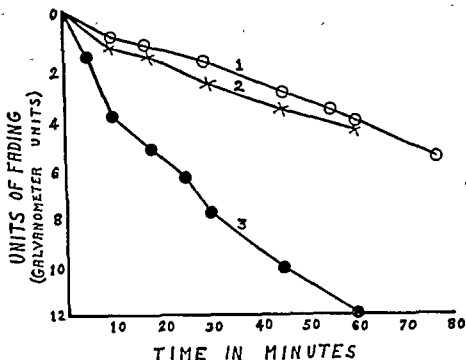


FIG. 1. THE RATES OF FADING OF COLORED SOLUTIONS PREPARED WITH CHLORAL HYDRATE ACCORDING TO THE TECHNIC OF THE ACCOMPANYING METHOD (SEE TEXT)

Curve 1 (hollow dots), method followed exactly; curve 2 (crosses), final dilution with 6 cc. instead of 5 cc. of alcohol; curve 3 (solid dots), final dilution with 6 cc. of distilled water instead of alcohol.

TABLE 1

Accuracy of method for determination of chloral hydrate

Solutions of chloral hydrate were added to whole blood or to protein free filtrates with the recoveries as shown.

	VOLUME	VOL. ADDED	CHLORAL ADDED	CONC. FILTRATE	FOUND IN FILTRATE	ERROR
	cc.	cc. sol.	mgm.	mgm./100 cc.	mgm./100 cc.	per cent
Blood	5	0.25	0.23	0.438	0.42	-4.1
	5	0.25	0.23	0.438	0.45	+2.7
	5	0.25	0.22	0.419	0.40	-4.5
	5	0.50	0.44	0.800	0.82	+2.5
	5	1.50	1.49	2.290	2.20	-3.6
	5	1.50	1.49	2.290	2.17	-5.2
Filtrate	20	0.05	0.22	0.214	0.20	-6.5
	20	0.10	0.44	0.432	0.43	-0.46
	20	0.20	0.88	0.871	0.88	+1.0
	20	0.30	1.32	1.300	1.24	-4.6

blood blank determination requires. Figure 2 shows that this is permissible with the expectancy of introducing a slight constant error into the determination of the absolute, but not relative amount, in a small percentage of such estimations.

Blank determinations were carried out on a large number of bloods known to be free of R-C-X₃ compounds. With the blank in the colorimeter, the light intensity was adjusted to a reading of 100 on the galvanometer. A tube of distilled water was then substituted for the blank and the galvanometer reading recorded. In more than 80 per cent of cases distilled water read within ± 0.5 of 96.5. This point depends upon clarity of reagents and individual colorimeter characteristics, and, it should therefore be determined in each laboratory by a small series of blank blood determinations. No difference in readings between the blanks of human and of dog bloods was observed, and no positive Fugiwara color was encountered except when the recent administration of an R-C-X₃ compound could be established. This latter point is contrary to the report of Mukerji and Ghose (7) but they may have obtained their slight positive

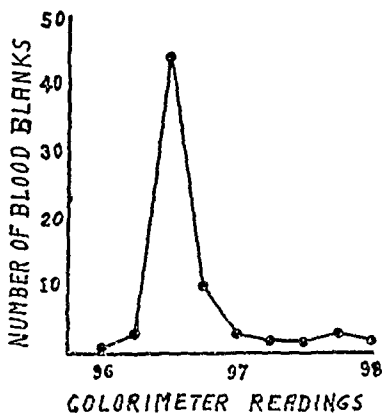


FIG. 2. THE GALVANOMETER READING OF DISTILLED WATER IN THE EVELYN COLORIMETER WHEN THE LIGHT INTENSITY IS SET AT 100 WITH A BLANK BLOOD DETERMINATION IN PLACE

from stop-cock grease, as discussed in section II above, or they may have been detecting the residual traces of previous doses of drugs, which as the author has pointed out (8), remain for a number of days in the blood stream.

REAGENTS. *Sodium tungstate solution*, 10 per cent,
Sulfuric acid, *N/12*. Prepare and standardize according to Haden (9).
Pyridine, *C.P.* This must be colorless.

Sodium hydroxide solution, 30 per cent. Filter through a fritted glass filter (Jena 3G3) at the time of preparation, and as necessary, to remove the precipitate which appears on standing. Store in a pyrex bottle.

Ethyl alcohol, 95 per cent.

METHOD. (1) Prepare a protein free filtrate from 1 cc. (or more) of blood by the Haden modification (9) of the Folin-Wu method. Centrifuge the mixture and filter through a No. 2 Whatman filter paper to obtain a maximum of clear filtrate. Exposure to air should be minimized by keeping the containers of blood and filtrate stoppered whenever possible.

(2) Deliver 3 cc. of 30 per cent NaOH into an Evelyn colorimeter tube (6) from a pinch-cock burette with a long tip. Add 1 cc. of colorless pyridine and stopper with a No. 3 rubber stopper.

(3) When all subsequent operations are in readiness, add 3 cc. of the blood filtrate; stopper lightly and mix by 5 seconds of lateral vigorous shaking.

(4) Place immediately in a well-regulated water-bath at 99°C. for exactly 60 seconds.

(5) Remove; stopper tightly, and place immediately in an ice-water bath for 60 seconds, then without removing from the bath,

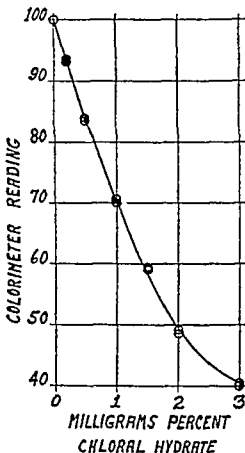


FIG. 3. CALIBRATION CURVE OF CHLORAL HYDRATE FOR THE EVELYN COLORIMETER

The concentrations of unknown solutions are read by reference of their galvanometer readings to this curve.

(6) Add 5 cc. of 95 per cent alcohol from a burette; stopper tightly, remove and invert two times to mix, and replace in the ice-water bath until the colorimeter reading is made.

(7) Within ten minutes, wipe the exterior of the tube dry and read immediately in the Evelyn colorimeter fitted with the No. 540 green filter.

Colorimeter adjustment. The initial light intensity is adjusted to a reading of 100 on the galvanometer, having in the colorimeter a blank determination of the blood taken prior to the administration of the drug to be determined. When this is not possible, the light intensity should be set with a tube of distilled water at a galvanometer reading of 96.5 (see series VI above).

Calculation. The concentration of the unknown in the filtrate, as read from a calibration curve (fig. 3), multiplied by 10 gives the mgm. per cent for whole blood. If the concentra-

THE RELATION OF PROTEIN INTAKE TO LEAD POISONING IN RATS

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Dietary protein has been shown to be a factor in determining the toxicity of hydrocarbons (1) cholic acid (2), iodoacetic acid (3), selenium compounds (4, 5) and sulfanilamide (6). The criteria of toxicity have been growth rate, mortality, gross and microscopic anatomy, and blood and urine composition. In general it has been found that high-protein diets and proteins of high quality protect animals better than low-protein diets and proteins of poor nutritional quality. This fact, together with the wide diversity of the toxic substances studied, suggests that protein protects through metabolic processes involving tissue repair rather than through more direct synthetic reactions resulting in detoxification. It is by no means certain that even mercapturic acids are formed directly from dietary cystine and bromobenzene (7). Reduced food intakes during the period when the toxic substances were fed accounts satisfactorily for the reduced growth rate observed in most cases and increased growth rates produced by supplements of cystine or cystine equivalents may have been simply improving the nutritional value of the diet which in nearly all cases was known to be deficient in cystine.

In the present study we have fed rats synthetic diets of different protein (casein) content containing lead chloride. In the *ad libitum* feeding experiments large differences in growth rates were found between groups receiving lead chloride and groups receiving the control diets. Corresponding differences in food intake were also observed. In order to eliminate this variable other groups of rats were fed the same diets using the paired feeding method. The results indicate that lead chloride exerts its effect chiefly by reducing food intake.

In seeking the cause of reduced food intake when lead chloride was consumed in the diet, we have supplemented the mixtures with cystine and methionine as well as with phosphate. All of these increased growth rates and food consumption. The amino acids, however, had the same effect when given without lead chloride and therefore probably exert their influence by

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improving the quality of the protein fraction. While we have no direct evidence of the effect of phosphate supplements in the basal diet the growth rate obtained without it was satisfactory, which does not support the assumption that the basal diet was deficient in phosphate. Phosphate did have a striking effect in reducing mortality and in supporting growth in a diet containing lead chloride. The results support the hypothesis that lead chloride produces a phosphate deficiency and confirms the work of Shelling (8).

In view of the importance of phosphate in the enzymes concerned with oxidation of metabolites, a phosphate deficiency might influence the oxygen uptake of tissues. This we have been unable to substantiate in our lead-poisoned animals. The oxygen uptake of liver slices was slightly accelerated in the rats that were fed *ad libitum* and slightly depressed in the pair-fed animals. Apparently lead chloride did not impair the enzymes of the liver, but the effect on other tissues such as bone marrow remains to be investigated.

All lead-poisoned animals showed lowered hemoglobin and red cells, increased stippled cells, and polychromatocytes. Studies on porphyrinuria in lead poisoning indicate that lead salts interfere with the formation of hemoglobin (9). That the quality of the protein consumed is also a factor in hemoglobin formation has been adequately proved (10). In our experiments rats exposed to inadequate protein intakes and lead chloride had a higher mortality, gained less weight and showed a greater decrease in hemoglobin than rats on adequate protein intakes.

While our experimental methods differed from those employed by some of the above-mentioned workers, it is quite probable that controlled food intake would show a similar dependence of growth responses upon food consumption in the presence of the other poisons studied.

EXPERIMENTAL. *Ad libitum feeding.* White rats (Osborne and Mendel strain) weighing about 65 grams were placed in individual cages and fed *ad libitum*. They were weighed every two days and food consumption was recorded after correcting for occasional spillage. Feeding was continued for about 35 days except in a few cases where the rats appeared unable to continue for that length of time. These were sacrificed in order to obtain blood and tissues for study. Some rats died very early in the feeding period.

Paired feeding. Litter mates of the same sex from our Wistar colony were kept on chow until they reached 65 grams in weight. They were then given our 20 per cent casein diet for one day in order to reduce intestinal contents and were then placed on the experimental diet. They were weighed every day and food consumption was determined after correcting for occasional spillage. The amount of basal diet given one of the pair was determined by how much the lead chloride-fed rat consumed. Feeding was continued for 25 days.

At the termination of the feeding periods all rats were killed by a blow on the head and blood from the heart was taken for hemoglobin determination by the Newcomer method. Samples were also prepared for counts of red cells, stippled cells, and polychromatocytes. Livers were divided, part being used for oxygen uptake measurement and part for histological study. Spleen and kidney were also preserved. The histological studies will be published later.

was fitted to rest on the clamp of the instrument which holds the liver plug stationary while slicing, and the smooth surface of the microtome aided in making smooth slices. Forty units on the scale raised the liver plug 0.2 mm. and gave the best slices. We believe that this is an improvement over free hand slicing though there is still considerable variability which we could not control.

The results showed that lead chloride feeding has a smaller effect on oxygen uptake than the uncontrolled variations in the tissues. There were no sig-

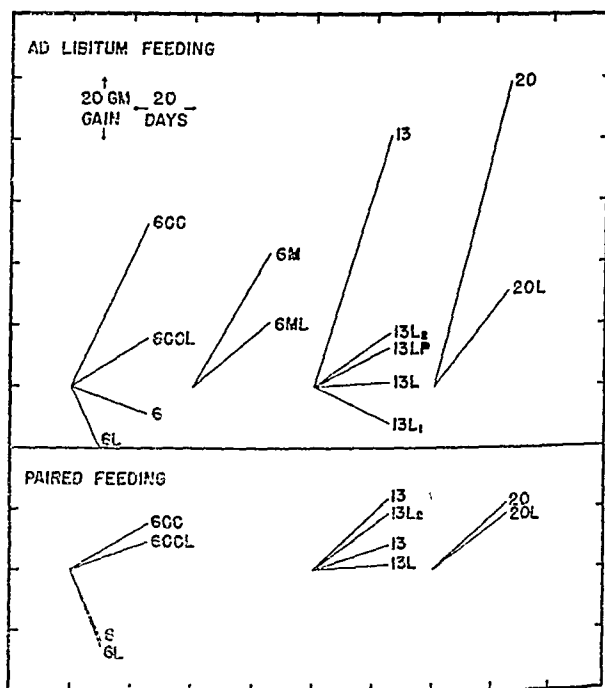


FIG. 1. AVERAGE GROWTH RATES FIRST 25 DAYS

nificant differences due to the composition of the diets. In the *ad libitum* group, lead chloride accelerated oxygen uptake slightly while in the paired feeding group the reverse was true. The mean QO_2 of 190 control slices from *ad libitum* fed rats was 7.87 with a standard deviation of 1.49. Two hundred and seventy-eight slices from lead chloride fed rats gave 8.49, st. dev. 1.52. The corresponding figures for the paired feeding groups were for controls 104 slices, mean 9.09 st. dev. 1.77, lead chloride rats 83 slices, mean 8.26 st. dev. 1.55.

Blood studies. Hemoglobin and red cells showed decided reductions in animals exposed to lead chloride. The lower values found in the *ad libitum* group are due to a slightly longer exposure period before the animals were sacrificed. Five rats receiving 6L diet *ad libitum* were killed after about one week and showed slight increases in hemoglobin and red cells. During this time they ate very little and lost considerable weight. It appears probable that these increases, which are the only exceptions noted, are due to decreased blood volume and do not represent actual increases in total hemoglobin or red cells.

Rats receiving cystine or methionine supplements in addition to lead chloride suffered smaller reductions of hemoglobin and red cells than those receiving unsupplemented lead chloride diets. It appears that these amino acids increase the efficiency of casein utilization for hemoglobin production as well as for growth.

The number of stippled cells and polychromatocytes per 10,000 red cells was greatly increased in all animals receiving lead chloride. Indeed not a single stippled cell was seen in any of the smears from rats on basal diets. The effects were less spectacular in the pair fed rats partly because of the shorter exposure period but partly also perhaps because of the difference in strain.

Acknowledgments. We gratefully acknowledge the assistance in hemoglobin determinations and red cell counts of Mrs. Anne Gerber and Mr. E. C. Thompson of the Pathology Section. Histological studies of the tissues of these rats will be published shortly. Dr. E. C. Hammond has been very helpful in the statistical studies. We are especially grateful to Dr. P. A. Neal for his interest and helpful criticisms throughout.

SUMMARY

1. Growth rates of white rats have been determined on synthetic diets containing 6, 13, and 20 per cent casein and on 6 per cent casein supplemented with 1 cystine and with *dl* methionine. The effect of adding 1.5 per cent lead chloride to these diets has been determined. Both *ad libitum* and paired feeding methods have been used.

2. Various levels of lead chloride and the effect of phosphate additions were studied in connection with 13 per cent casein diet.

3. Oxygen uptake of liver slices, hemoglobin, red cells, stippled cells, and polychromatocytes were also studied.

4. The toxicity of lead chloride as indicated by mortality, growth rate, and hemoglobin level is affected by the quantity and quality of the protein content of the diet.

5. Oxygen uptake of liver slices is not greatly affected by lead chloride feeding nor by variation in the protein content of the diet.

6. Several interpretations of the effect of lead chloride on growth are

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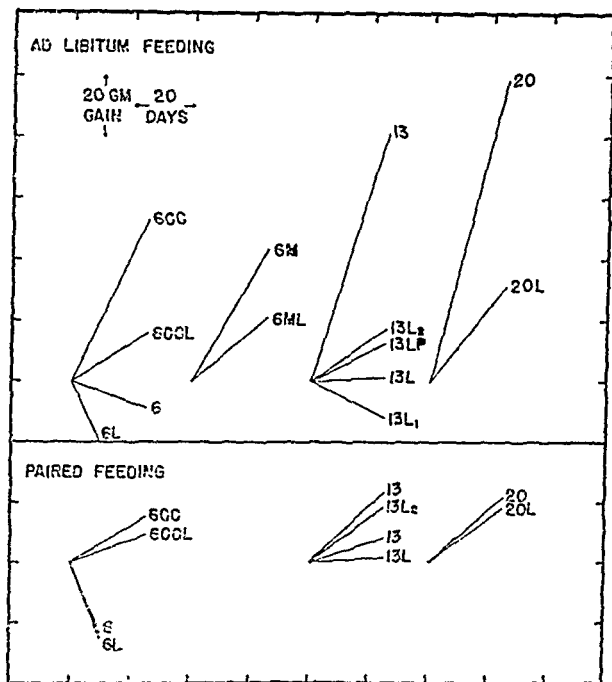


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5. Oxygen uptake of liver slices is not greatly affected by lead chloride feeding nor by variation in the protein content of the diet.
6. Several interpretations of the effect of lead chloride on growth are

possible from this data. Lead chloride reduces growth rate (a) by reducing food intake, (b) by removing phosphate from the diet, (c) by diverting amino acids from use for growth to use for rebuilding hemoglobin.

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STUDIES ON THE TOXICITY OF ACTINOMYCIN

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Of the various bacteriostatic and bactericidal substances isolated from soil microorganisms (1, 2, 3), actinomycin appears to be of particular interest in view of its marked *in vitro* activity against gram-positive, and to some extent against gram-negative microorganisms. This substance was isolated in a pure state, by the use of the chromatographic absorption method, and crystallized (4). Although it was found to contain about 13 per cent nitrogen, it is not a protein and has no free amino group. It may be considered as a polycyclic nitrogen compound. Actinomycin was shown (5) to be highly toxic to animals. In this communication data are presented which deal further with its toxicity, as well as with the pharmacology and bactericidal properties of this substance.¹

METHODS. Swiss mice of the CFl strain and albino rats of the Wistar strain were used throughout this investigation. The rabbits employed were obtained from various sources and therefore were not of a uniform strain. All animals were maintained on a balanced diet with sufficient water available at all times. A total of 690 mice, 90 rats and 25 rabbits were used in this study.

The strains of streptococci and pneumococci used in the *in vivo* studies were of known mouse virulence and were supplied through the kindness of Dr. Wm. Feirer of Sharp & Dohme, Inc., Glenolden, Pa. These organisms were grown for 6 hours in meat infusion broth containing 10 per cent rabbit blood, and the virulence was maintained by frequent mouse passages. The cultures of *Clostridium welchii* and *Cl. tetani* employed in the *in vitro* studies were grown in brain heart infusion media containing 0.1 per cent agar.

Solutions of actinomycin were prepared by dissolving 10 mgm. of the dry substance in 1 cc. of warm 95 per cent ethyl alcohol and diluting in sterile distilled water or physiological salt solution to the desired concentration. Control experiments were conducted with similar concentrations of alcohol.

RESULTS. *Acute toxicity.* This was determined in mice, rats and rabbits. Varying doses were given intravenously, intraperitoneally, subcutaneously

¹ The actinomycin A used in these studies has been largely prepared by Dr. Max Tishler of the Research Laboratories of Merck & Co. Inc. to whom the authors are indebted.

and orally. Animals dying during the first 7-day period were autopsied and the tissues retained for histological study.² Only the results obtained in mice are presented in table 1.

From these data it is evident that actinomycin is extremely toxic for mice. Doses of 1 mgm. per kilogram were found to be lethal to other animal species when given intravenously, intraperitoneally or subcutaneously. This toxicity is more apparent when observations are extended over a 7-day period.

The first signs of toxicity appear 5 to 6 hours after intravenous or intraperitoneal injection and 10 to 12 hours following subcutaneous or oral administration. They consist of weakness, languor, anorexia and diarrhea. Gross

TABLE 1
Acute toxicity of actinomycin in mice

DOSE	NUMBER OF MICE PER DOSE	NUMBER OF DEAD MICE							
		Intravenous		Intraperitoneal		Subcutaneous		Oral	
		A*	B*	A	B	A	B	A	B
<i>mgm./kgm.</i>									
0.15	10	0	0	0	0	0	0		
0.25	10	0	2	0	2	0	1		
0.50	10	0	8	0	10	0	10		
1.0	10	9	10	0	10	0	10		
1.5	10	9	10	7	10	4	10	0	0
2.0	10	10	10	8	10	10	10	0	0
2.5	10	10	10	10	10	10	10	0	0
5.0	10	10	10	10	10	10	10	0	0
10.0	10							0	3
15.0	10							0	7
20.0	10							10	10
30.0	10							10	10

* A = 1 day observation; B = 7 days observation.

hematuria occurred frequently in rabbits from 4 to 6 hours after intravenous administration of 1 mgm. of actinomycin per kilogram body weight. As intoxication progressed the nervous system became involved, as evidenced by ataxia and tonic convulsions. The immediate cause of death appeared to be respiratory failure, since the heart continued to beat for some time after respiration ceased.

Upon macroscopic examination, evidence of congestion in the lungs, liver, spleen and intestinal viscera was found. The most striking finding, however, was the diminution in the size and weight of the spleen. On a weight basis,

² Histological findings will be reported elsewhere by Dr. Wm. Antopol, Chief Pathologist of the Beth Israel Hospital, Newark, N. J.

the spleens of mice receiving a single dose of actinomycin were approximately one-half the normal size.

Chronic toxicity studies. The effect of prolonged administration of small amounts of actinomycin to animals was investigated in a series of 60 mice and 30 rats by injecting intraperitoneally varying doses in the form of a sterile aqueous solution. The concentration of the solution was adjusted so that each animal received 0.25 cc. per 20 grams body weight. Observations were made twice daily for the duration of the experiment. These experiments are summarized in table 2.

It is evident that doses as small as 1 microgram of actinomycin per 20 grams of animal weight will produce death in most mice and rats within 10 days. Evidence of toxicity began to appear after one or two days of treatment. At this time there were signs of general debility and muscular

TABLE 2
Chronic toxicity of actinomycin in mice and rats

DOSE	NUMBER OF MICE	SURVIVAL—DAYS									
		1	2	3	4	5	7	11	15	20	30
mgm /kgm.											
0.025	20	20	20	20	20	20	19	19	19	15	15
0.050	20	20	20	20	19	18	2	0	0	0	0
0.100	20	20	20	20	19	9	0	0	0	0	0
	NUMBER OF RATS										
0.025	10	10	10	10	10	10	10	6	4	2	0
0.050	10	10	10	10	9	8	5	0	0	0	0
0.100	10	10	10	10	4	0	0	0	0	0	0

weakness without significant loss of weight. An occasional rat developed gross hematuria, but this was not evident in mice. Prior to death the body temperature of rats was found to be subnormal ranging from 32–33° centigrade. As in the acute experiments, death appeared to be due to respiratory failure.

The gross pathological results were essentially the same for mice and rats. After 3 or 4 doses evidence of hydrothorax and marked ascites appeared in most animals. Congestion of the abdominal viscera, liver, kidney and lungs was more apparent than following a single dose of actinomycin. In all animals a marked decrease in both the size and weight of the spleen was found which in some cases was approximately one-tenth the weight of the average normal spleen.

Effect on liver and kidney function. The hepatic function of 5 rats was measured before and after the administration of actinomycin by the micro

method of Seeler and Kuna (6). Doses of 0.5 microgram actinomycin per 100 grams of body weight were given intraperitoneally to rats daily and hepatic function was measured every third day over a period of fifteen days. The results indicate that a single dose of actinomycin produced some liver damage in 2 out of 5 rats. After 10 days, however, liver damage was present in all rats as evidenced by a dye-retention of 20 to 40 per cent.

By means of the method described by Burns (7) the effect of actinomycin upon water diuresis was studied in rats. The procedure consisted in measuring the water diuresis of a series of 20 rats before and after administration of actinomycin. The rats were divided into groups of 5 and fed 5 cc. of warm tap water per 100 grams body weight. Immediately following this a dose of 25 micrograms of actinomycin per kilogram body weight was given intraperitoneally. The amount and rate of urine excreted was measured with the diuresis recorder described by Kniazuk (8). Analyses of the urine (albumin, specific gravity) were performed at the beginning and end of the experiment. Under the conditions of this experiment actinomycin depressed the water diuresis significantly. No studies were undertaken to determine the cause or nature of this depression. Examination of the urine showed a very slight trace of albumin in all groups but no significant change in the specific gravity was found.

Effect on blood. The action of actinomycin on the blood was investigated in rats and rabbits. Red, white and differential counts were made before and after administration of actinomycin. In addition, in the rabbit experiments the volume of red cells and the hemoglobin were estimated. After the normal values were established a blood examination was made 5 hours after administering 100 micrograms of actinomycin per kilogram. The drug was administered daily and counts made every other day for 10 additional days. The results of these experiments indicate that actinomycin produces a slight leukocytosis without significant changes in the red or differential count.

Examination of the effect of actinomycin on erythrocytes *in vitro*, studied in both liquid and solid media, showed that it is not hemolytic as reported for tyrothricin (9).

Effect on circulatory and respiratory systems. The carotid blood pressure was measured in rabbits and cats under urethane anesthesia (1.2 gram per kilogram intramuscularly) by the usual manometric method. Injections of actinomycin dissolved in saline were made into the jugular vein and the leg volume, and blood pressure and respiratory movements recorded. Contrary to expectations, doses as large as 30 mgm. per kilogram (30 times the 24-hour lethal dose) administered over a period of 10 minutes had no immediate effect. Moreover, no marked changes were found over an observation period of 5 hours. In some animals 30 mgm. per kilogram caused a slight rise in blood pressure without significantly affecting leg volume.

Antibacterial efficacy in vitro. The bactericidal and bacteriostatic activities

of actinomycin were determined by means of the standard F.D.A. phenol coefficient technique (10) and a modification of Kolmer's bacteriostatic method (11). In this manner both the bacteriostatic and bactericidal actions were determined. The absence of turbidity in the drug-bacteria mixtures at the end of each 24 hour period was taken as an indication of inhibition. With these methods, applied to strains of *Staph. aureus*, *Cl. welchii*, *Cl. tetani*, *Strep. hemolyticus* and type I pneumococcus, actinomycin was found to be a powerful bacteriostatic agent for both anaerobic and aerobic gram-positive microorganisms. Dilutions of 1:1,000,000 were bactericidal for the streptococci and pneumococci and dilutions of 1:2,000,000 were bacteriostatic. In order to produce a similar effect upon the anaerobic microorganisms concentrations of 1:60,000 to 1:100,000 were found necessary. Over a 3 to 4 day period this bacteriostatic action slowly became bactericidal. Actinomycin appears to be somewhat more effective against pneumococci and streptococci than against staphylococci. This is similar to the findings reported for tyrothricin (12).

A procedure for measuring antibacterial activity of actinomycin in solid media, such as agar, has been described previously by Waksman and Woodruff (13). The antibacterial activity of actinomycin against aerobic pathogenic microorganisms established the fact that it is a more effective bacteriostatic agent in solid than in liquid media, inhibiting the growth of *Strep. hemolyticus* in the former in concentrations of 1:4,000,000. Waksman and Woodruff reported (13) inhibition of certain cocci in as low a concentration as 1:100,000,000.

A series of similar experiments conducted in the presence of 10 per cent rabbit serum indicated that the latter has no effect upon the action of actinomycin.

Antibacterial action in vivo. The antibacterial action of actinomycin *in vivo* was studied in mice infected with a mouse-virulent strain of type I pneumococcus and a hemolytic strain of streptococci Lancefield group A. Mice were inoculated intraperitoneally with 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} dilutions of a six hour culture grown at 37°C. In both the streptococcus and pneumococcus infections, the foregoing dilutions were equal to approximately 10,000, 1,000, 100 and 10 lethal doses of organisms respectively. The M.L.D. was determined by both the titration test and by plate count. The infected mice were treated immediately after the inoculation with single doses of 0.25, 0.5 and 1.0 microgram of actinomycin intraperitoneally, subcutaneously or orally. A second series of experiments consisted of infecting mice intraperitoneally and treating them orally, subcutaneously or intraperitoneally with doses of 0.1 and 0.2 microgram every 4 hours until death. Actinomycin afforded little or no protection under these conditions (table 3). In a control group of similarly infected mice, 8 micrograms of gramicidin afforded complete protection.

Antiprotozoic action. The antiprotozoic action of actinomycin was deter-

mined by the use of a procedure similar to the above. Mice were inoculated intraperitoneally with 0.25 cc. of citrated saline containing approximately 100 organisms of *Trypanosoma equiperdum*, and then immediately given an intraperitoneal or subcutaneous injection of 0.1, 0.2 and 0.5 microgram actinomycin. Observation was made over a 10 day period. Under these conditions, actinomycin afforded some protection when injected intraperitoneally although no protection was found by subcutaneous administration (table 3).

Distribution of actinomycin in body tissues. The extreme activity of actinomycin *in vitro*, with or without the presence of blood serum, contrasted with its apparent complete lack of activity *in vivo* and raised the question as to its fate in the organism. The highly tinctorial properties of actinomycin together

TABLE 3

Action of actinomycin in vivo in streptococcal and trypanosoma infections in mice

SUBSTANCE	A. STREPTOCOCCUS INFECTIONS—100 LETHAL DOSES										
	Number of mice	Dose, micrograms per mouse	Number surviving in days								
			1	2	3	4	5	6	7	8	9
Gramicidin.....	40	8.0	40	40	38	28	25	25	25	25	25
Actinomycin.....	40	1.0	4	0							
Actinomycin.....	40	2.5	2	0							
Actinomycin.....	40	5.0	0	0							
Actinomycin.....	40	10.0	0	0							
Controls.....	40		0	0							
	B. TRYPANOSOMA EQUIPERDUM—1 LETHAL DOSE										
			1	2	3	4	5	6	7	8	9
Bayer 205.....	20	5,000	20	20	20	20	20	20	20	20	20
Actinomycin.....	20	0.2*	20	20	20	14	11	6	3	1	0
Controls.....	20		20	20	16	10	2	1	1	1	0

* Dosed daily for 8 days.

with its marked specific bacteriostatic activity can be utilized to determine its distribution in the animal body. Preliminary studies were made in mice, rats and rabbits using the following technique: After intravenous or intraperitoneal injections of actinomycin, 0.75 cc. of blood was removed from the animal by cardiac puncture at exactly 1, 5, 15 and 60 minutes following injection and placed in a sterile glass vial containing 2 mgm. of dry sodium oxalate. Quantities of 0.1, 0.2 and 0.4 cc. of the blood were added to 2 cc. of warm (45°C.) nutrient agar, which was then poured into small petri dishes and allowed to solidify. Shortly after this, cultures of *Streptococcus hemolyticus*, *Staphylococcus aureus*, *Sarcina lutea* and *Bac. mycoides* were streaked upon the surface of the blood agar and the plates incubated at 37°C. for 24 and 48 hours. Control experiments were conducted with normal blood

removed from the animal prior to actinomycin administration and also with blood to which known amounts of actinomycin had been added. In this manner the degree of inhibition obtained with blood taken from animals injected with actinomycin could be compared with control blood samples. Similar experiments were conducted with urine collected from anesthetized rabbits by sterile catheterization of the ureters over a period of 6 hours. The results of these experiments indicate that a dose of 2.5 micrograms of actinomycin per 20 grams given intravenously disappears from the circulating blood within a period of 15 minutes. Analysis of the blood at later time intervals showed no inhibitory effect.

Colorimetric analyses of ether extracts of urine indicated that within a period of 6 hours from 10 to 20 per cent of actinomycin is excreted in the urine of rabbits.

DISCUSSION. The foregoing experiments show actinomycin to be a powerful bacteriostatic and bactericidal agent *in vitro*. Moreover, the presence of serum does not diminish the efficacy of this substance as has been reported for other soil agents such as tyrocidin, gramicidin and tyrothricin (14). On the other hand, essentially no protection is afforded to mice inoculated with cultures of *Streptococcus hemolyticus* or pneumococcus type I. The lack of activity *in vivo* may be due, among other things, to the rapid disappearance of actinomycin from the blood. The present evidence suggests that this removal is due to renal excretion and tissue absorption, since a large proportion of the drug can be recovered from all tissues as well as from the urine. In spite of the rapid disappearance of actinomycin from the blood, this agent is extremely toxic to all animal species, death apparently resulting from respiratory failure. Most deaths do not occur until 15 to 20 hours after actinomycin inoculation. In this respect actinomycin is similar to certain toxins such as ricin. The fact that large doses of actinomycin can be injected intravenously into rabbits without producing any visible effects upon the blood pressure or respiratory movements indicates that high blood concentrations of the drug can be tolerated by the higher nervous centers. This suggests that actinomycin does not cause death by acting directly on the higher nerve centers, but rather through certain pathological changes which eventually involve the respiratory system.

SUMMARY

1. Doses of 1.0 mgm. or more of actinomycin per kilogram are lethal for mice, rats and rabbits when administered intravenously, intraperitoneally, subcutaneously or orally. The toxicity is more evident when observations are extended over a seven day period.

2. Doses as small as 50 micrograms per kilogram intraperitoneally produce death in mice or rats when administered daily over a six-day period. Death

s accompanied by severe gross pathological changes, notably a marked shrinkage of the spleen.

3. Liver and kidney function appear to be impaired following daily administration of actinomycin.

4. Intravenous injection of actinomycin produces no significant changes in blood pressure or respiration.

5. Actinomycin is markedly bacteriostatic *in vitro* for both aerobic and anaerobic pathogenic bacteria.

6. *In vivo*, actinomycin affords almost no protection to mice inoculated with *Streptococcus hemolyticus* or pneumococcus type I. Some effect is found in *Trypanosoma equiperdum* infections.

7. Analysis of the fate of actinomycin upon intravenous injection indicates that it is rapidly removed from the blood and is found in various quantities in all organs of the body. Rabbits excrete 10 to 20 per cent of the actinomycin 5 to 6 hours after injection.

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PEPTONE SHOCK IN RABBITS

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The remarkable similarity between peptone shock and anaphylactic shock in dogs was emphasized by Biedl and Kraus (1) in 1909. Subsequent studies (2, 3, 4, 5, 6) have demonstrated that this similarity in symptomatology is based upon a similarity in pathogenesis in that the dominant symptoms of both reactions can be attributed to a liberation of histamine from the tissues of the animal. One of the indications of this is a striking increase in the blood histamine. Rose and Weil (7) found that anaphylactic shock in rabbits is characterized by a prompt decrease in the total blood histamine, an observation which has been confirmed in our laboratory (7). This apparently paradoxical behavior of the rabbit does not necessarily imply that histamine is unrelated to the symptoms of anaphylaxis in this animal. It is possible that this reduction in the total blood histamine may only be an experimental verification of the participation of histamine in anaphylaxis in the rabbit, since Katz (9) has shown that the addition of antigen to the blood of sensitized animals leads to the release of histamine from blood cells to plasma, so that histamine would thereby be freed to produce its effects and *pari passu* disappear from the blood as it diffused into the tissues. An additional factor accounting for the reduction in total blood histamine in the rabbit, is the marked leucopenia which follows the injection of antigen, as Code (10) has shown that the major portion of the blood histamine of rabbits is contained in the leukocytes.

Irrespective of the merits of the argument as to the rôle of histamine in anaphylaxis in the rabbit, the findings with respect to changes in the concentration of histamine in blood and plasma provide an objective method by which the analogy between peptone shock and anaphylactic shock can be investigated. It is a reasonable hope that some of the features of anaphylaxis may be clarified as progress is made in determining whether they are specific to anaphylaxis or are common to other and less complex reactions. Bally (11) has shown that the two reactions in the rabbit are similar with respect to the gross symptomatology (circulatory effects, blood coagulation, etc.). The present report therefore is concerned with the changes in the blood histamine of intact rabbits subjected to peptone shock and with the effects of peptone when added to rabbit's blood *in vitro*.

EXPERIMENTAL. Large, white female rabbits were anesthetized with pentobarbital and arranged for recording the carotid blood pressure. Blood samples were obtained by cardiac puncture for leukocyte counts and for estimation of the histamine content by Code's (12) modification of the method of Barsoum and Gaddum. The peptone solution was injected *via* the femoral vein in a dose of 2 cc. per kilogram. We employed a 20 per cent solution of Bacto-Protone (Difco) prepared by dissolving the peptone in boiling water, acidifying and shaking the solution with permutit, and then filtering and neutralizing it prior to use. This procedure removes practically all of any contaminating histamine which may be present. For the *in vitro* experiments, blood was obtained by cardiac puncture from unanesthetized rabbits, kept from clotting by means of heparin and divided into three 5 cc. samples. The total blood histamine was determined on the first sample. The second sample was centrifuged and the plasma histamine determined; 0.2 cc. of the peptone solution was added to the third sample, mixed by inverting the tube once, immediately centrifuged, and the plasma histamine determined. The total blood histamine of blood samples to which the peptone had been added was determined in several instances to prove that the peptone solutions were free of histamine. The results are shown in the tables.

DISCUSSION. As noted in table 1, the intravenous injection of proteoses in normal rabbits results in a prompt reduction of the blood histamine and a leukopenia similar to that produced by the injection of antigen into previously sensitized animals. As shown in table 2, there is also a release of histamine from blood cells to plasma when peptone is added to heparinized blood; a similar change as occurs when antigen is added to heparinized blood from sensitized animals. With reference to the reactions in the intact animals, our observations agree with those of preceding workers in that the general symptoms from peptone are usually not as severe as those seen in anaphylactic experiments. Correspondingly Rose (13) found in anaphylactized rabbits that the blood histamine was decreased for 3 to 4 hours, while in our experiments the histamine values were nearly back to normal in 1 hour. With reference to the *in vitro* experiments some additional comment seems appropriate. Reasoning from various observations by Tarras-Wahlberg (14) and Code (10) it seems probable that there may be no free histamine in the circulating plasma of the normal intact rabbit, and that injury to the blood incidental to its collection, centrifugation, etc., leads to some release of histamine from cells to plasma. This is extremely marked, for example, if coagulation is permitted, and thus fresh rabbit serum has a very high content of histamine. When rabbit blood is collected and handled carefully and kept from clotting by means of heparin the histamine content of the plasma obtained after centrifugation is comparatively low, but the histamine values of this so-called normal plasma may well represent some unavoidable damage to the cellular elements. Be that as it may, the extremely rapid and marked

increase in plasma histamine which can be produced by the addition of antigen to the heparinized blood of sensitized animals, or by the addition of proteose to the heparinized blood of normal animals, is far beyond that which occurs incidentally to the experiment. In the experiments reported here, the control

TABLE 1

Blood histamine and leukocytes before and after peptone shock in rabbits

EXPERIMENT	BEFORE		AFTER						DEGREE OF SHOCK
	Hist.	W.B.C.	10 minutes		20 minutes		60 minutes		
			Hist.	W.B.C.	Hist.	W.B.C.	Hist.	W.B.C.	
1	3.3	17,600	2.5	2,900					Moderate
2	5.0	6,400	5.0	800	1.1	6,200	4.3	6,600	Moderate
3	5.3	8,400	2.6	3,200	2.0	4,000	6.0	7,800	Moderate
4	4.0	6,600	1.3	4,000	1.3	3,200	3.7	5,400	Moderate
5	2.6	5,200	0.6	800	1.0	1,400	3.0	4,800	Severe
6	4.6	8,200	2.0	6,000	1.0	1,200	3.3	5,800	Mild
7	4.6	9,400	1.1	8,200	1.2	1,400	3.3	7,800	Moderate
8	5.0	7,400	2.6	4,000	1.6	3,200	4.3	6,800	Moderate

Hist. = histamine values in micrograms histamine base per cc. of blood.

W.B.C. = leucocytes per cu. mm. of blood.

TABLE 2

Transfer of histamine from cells to plasma as result of adding peptone to heparinized rabbit's blood in vitro

EXPERIMENT	CONTROL 5.0 cc. BLOOD + 0.2 cc. SALINE			TEST 5.0 cc. BLOOD + 0.2 cc. 20 PERCENT BACTO PEPTONE		
	Total histamine*	Cell histamine†	Plasma histamine‡	Total histamine*	Cell histamine†	Plasma histamine‡
1			1.5			3.0
2			2.0			6.2
3	11.0	8.25	2.75	10.0	4.5	5.5
4	16.5	15.0	1.5	17.0	10.5	6.5
5	20.0	16.75	3.25	18.0	11.5	6.5

* Histamine equivalent (in micrograms of base) in 5 cc. of blood by assay.

† Cell histamine—by difference between plasma histamine and total histamine.

‡ Plasma histamine = plasma histamine per cc. of plasma by assay \times cc. of plasma in each case (from hematocrit).

and peptone-treated samples of blood were aliquots of the same specimen, were otherwise handled identically, and were centrifuged simultaneously. The already-established extensive parallelism between the effects of proteoses in normal animals and the effects of antigen in sensitized animals is thus extended.

CONCLUSIONS

1. The intravenous injection of proteose solutions into rabbits leads to a prompt reduction in the total blood histamine and to a leukopenia.
2. The addition of proteose solutions to heparinized rabbit's blood leads to a rapid release of histamine from cells to plasma.
3. Both of these effects are similar to those occurring in anaphylaxis in the rabbit.

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STUDIES ON THE FATE OF MORPHINE¹

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The fate of morphine in morphine addicts has not been fully determined. Most of the present knowledge regarding the morphine content of various biological materials has been derived from analyses of morphinized laboratory animals which usually had received considerably more morphine per kilogram of body weight than is taken by human addicts. It was thought that the determination of the morphine concentration in such biological materials as urine, feces, blood, liver, saliva, bile, gastric contents, and perspiration of human subjects might offer additional information regarding the distribution and excretion of this opium alkaloid.

METHODS AND MATERIALS. Most of the materials used were obtained from patients who had active physical dependence on morphine at the time of the examination. Samples of liver were obtained from patients brought to autopsy. Bile was collected from a T-tube in the common duct of a patient who had had a cholecystectomy and cholelithotomy. This patient, a former addict, had been receiving 90 mgm. morphine sulphate daily for 3 days but had not developed physical dependence.

Urine. It was necessary to modify the morphine-molybdate-vanadate procedure for the determination of small amounts (0.03 to 0.2 mgm.) of morphine (1, 2), for the final extraction residue must be comparatively free of interfering substances. The urine was treated in a liquid-liquid extractor and the residue obtained from this extraction was dissolved in about 50 cc. of a saturated solution of sodium bicarbonate and re-extracted in another liquid-liquid extractor. If the residue appeared to be reasonably free of excessive amounts of interfering substances, the second liquid-liquid extraction was omitted. The residue obtained from this extraction was dissolved in 0.5 per cent hydrochloric acid and transferred to a separatory funnel. This solution was extracted once with ethyl alcohol-chloroform mixture (1-3) after which the extraction solvent, containing some of the interfering substances, was discarded. Then the acidic, aqueous solution was saturated with sodium bicarbonate to liberate the alkaloidal base. This was extracted 3 times in a separatory funnel with the ethyl alcohol-chloroform mixture;³

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³ Ethyl acetate may be used instead of the ethyl alcohol-chloroform mixture. In order not to carry over any urine adhering to the sides and stem of the funnel, the ethyl acetate was decanted from the top of the separatory funnel onto a dry filter paper in an ordinary funnel. The filter paper serves to absorb suspended urine droplets and insoluble matter. Each portion of the ethyl acetate extraction was saved, transferred to an

the combined extraction solvent and 0.3 cc. of 0.5 per cent hydrochloric acid were transferred to an evaporating dish and evaporated to dryness. The residue should now be suitable for the preparation and formation of the morphine-molybdate-vanadate complex.

The residue containing less than 0.2 mgm. of morphine was dissolved in 1.5 cc. of water and 0.2 cc. of 2.6 N sulfuric acid. To this solution was added 0.2 cc. of 10 per cent ammonium molybdate which further precipitated impurities. After standing 10 to 15 minutes it was filtered through a wet Whatman No. 42 filter paper. The filtrate and washings were expected to be colorless and water clear and not to exceed 3 cc. in volume. The addition of 0.1 cc. of 2 per cent filtered ammonium vanadate produced a light straw-colored solution which gradually faded on standing. The morphine-molybdate-vanadate complex developed as a fine, white, suspension which finally was compared nephelometrically with a standard morphine solution treated in a similar manner. Before reading the turbidity in a Bausch and Lomb nephelometer, the standard and unknowns were first diluted to 15 cc.

Occasionally some difficulties are encountered with the above procedure. A residue containing large amounts of impurities often produced a voluminous precipitate following the addition of the ammonium molybdate. It was sometimes necessary to filter this twice before a clear filtrate was obtained. Occasionally a blue-green color developed in the solution while it was standing for the precipitate to form; this color was due to certain impurities having strong reducing properties. The addition of ammonium vanadate to the blue-green filtrate generally did not produce a turbidity, even when morphine was known to be present. This shows that it is essential to be careful in preparing the urinary residue before the morphine-molybdate-vanadate test is made.

Hydrolysis of the bound morphine in urine was carried out as previously described (2). Five parts of urine and 1 part of concentrated hydrochloric acid were boiled for 3 hours under a reflux condenser. After cooling and filtering the solution was saturated with powdered sodium bicarbonate and extracted by the usual procedure. For qualitative analysis it proved to be advisable to boil acidified urine three hours prior to the liquid-liquid extraction procedure, in order to liberate any bound morphine and thereby to increase the sensitivity of the test.

Feces. Two grams of dried powdered feces were placed in a 250 cc. Erlenmeyer flask containing exactly 100 cc. of saturated sodium bicarbonate. This was mixed thoroughly and allowed to soak for at least 1 hour, shaking the flask and contents at frequent intervals. The material was then centrifuged and the supernatant fluid decanted. A suitable aliquot, usually 70 cc. of the solution, was placed in a liquid-liquid extractor and treated in the same manner as urine. After evaporation of the extraction solvent the residue was dissolved in 0.5 per cent HCl and the solution extracted twice in a separatory funnel with the ethyl alcohol-chloroform mixture to remove soluble substances. Since morphine is not extracted from an acid solution, the extraction solvent was discarded. Then the aqueous, acidic solution was saturated with powdered sodium bicarbonate and re-extracted 3 times with the ethyl alcohol-chloroform mixture. The combined ethyl alcohol-chloroform mixture was evaporated to dryness. The residue was analyzed for morphine by the molybdate-vanadate procedure outlined for urine.

Hydrolysis of bound morphine in feces was carried out by boiling 2 grams of feces,

evaporating dish, and evaporated to dryness. When excessive amounts of colored matter were present in the residue, it was dissolved in 0.5 per cent hydrochloric acid, transferred to a separatory funnel, saturated with sodium bicarbonate, and re-extracted 3 times with ethyl acetate.

50 cc. water, and 10 cc. concentrated HCl for 3 hours under a reflux condenser. After the mixture had cooled, 40 cc. of water were added, the contents centrifuged, and the supernatant fluid filtered. The filtrate, saturated with powdered sodium bicarbonate, was placed in a liquid-liquid extractor and treated the same as urine. One extraction in the liquid-liquid extractor was usually sufficient for feces.

Liver and blood. Liver was ground up in a meat grinder. Both the ground liver and the blood were then treated alike. To each was added an equal weight of water followed by the slow addition of 4 parts by weight of 10 per cent trichloroacetic acid. The mixture was then heated slowly on a water bath for an hour, after which it was filtered.

TABLE 1

Morphine concentration in biological materials from morphine addicts

MATERIAL	NUMBER OF SPECIMENS	AMOUNT OF MATERIAL USED FOR ANALYSIS	DAILY DOSAGE	MORPHINE CONCENTRATION				RATIO OF BOUND TO FREE
				Free		Bound		
				Average	Range	Average	Range	
		cc.	mgm.	mgm. per cent	mgm. per cent	mgm. per cent	mgm. per cent	
Urine.. . . .	5	50-70	8-20*	0.15	0.12-0.18	0.27	0.20-0.34	1.61
	2	65	100	0.36	0.31-0.41	0.84	0.70-0.98	2.33
	1	65	168	0.61		1.69		2.77
	1	65	200	0.48		1.25		2.60
	1	65	487	0.97		6.26		6.45
	1	10	1,180	1.26		16.50		13.10
	1	5	2,072	1.24		22.50		18.15
		grams						
Feces (dried)..	9	2	283-489	0.90	0.45-1.67	0.177	0.0-0.57	0.21
	1	1	2,455	5.88		0.0		
		cc.						
Blood	5	7-50	200-600	0.0		0.0		
Saliva	6	20-260	105-4,200	0.0		0.0		
Gastric content.....	4	55-70	120-200	0.04	0.02-0.07	0.06	0.0-0.13	1.50
Perspiration....	3	58-215	300	0.18	0.02-0.5			
Liver.	2	100-200	290-670	0.09	0.02-0.16			
Bile.	2	50-70	90	0.0		0.07		

* Single injections, non-tolerant individuals.

The filtrate was evaporated to a volume suitable for extraction in a liquid-liquid extractor.

In analyzing blood for bound morphine a suitable portion of the trichloroacetic acid filtrate was treated in the same manner as urine.

Saliva, perspiration, bile, and gastric contents. For the determination of free and bound morphine these materials were treated the same as urine. It was necessary to make 2 successive extractions in the liquid-liquid extractor on bile.

RESULTS. The results are shown in table 1. The amount of bound morphine excreted in urine varied from 3 to 10 times that of the free, both being greater with the higher dosage. The average daily percentages of free and

bound morphine excreted in the urine were approximately 6 and 30, respectively.

The amount of free morphine found in dried feces varied with the dosage. The average daily amount excreted by the bowel was less than 1 per cent of the daily dose, which is much less than that excreted by the kidney. If bound morphine occurs at all in the feces, it is in very small amounts.

Both free and bound morphine were found in gastric contents, the amount of bound being approximately twice that of the free in a man receiving 160 mgm. morphine sulphate daily. No morphine was found in the vomitus of two morphine addicts on the second day of withdrawal.

Small amounts of bound morphine were found in the bile from a non-tolerant patient receiving 15 mgm. morphine sulphate every 4 hours following an operation.

No morphine, either free or bound, was found in blood or saliva.

Small amounts of free morphine were found in perspiration and liver. These materials were not studied for the presence of bound morphine.

DISCUSSION. It is not yet known with what substance morphine is conjugated, but since morphine contains an hydroxyl group comparable to the phenols, it is probable that the drug is excreted largely as a conjugate of glucuronic acid or its lactone form, glucurone. Further evidence is being presented (3) in support of this hypothesis.

A number of investigators (4, 5, 6, 7) have shown that morphine is present in the gastric contents of morphinized animals. No study has come to our attention, however, in which bound morphine has been found in gastric contents of addicted humans. Apparently this form of morphine is either re-absorbed and excreted in a free form, or is hydrolyzed as it passes through the intestinal tract, for only free morphine was found in the feces. The bound morphine in the bile may follow the same course as that in the gastric contents.

As far as is known, this is the first time that it has been shown that morphine is excreted in the perspiration of morphine addicts. The amount excreted is low as compared with that in urine.

The failure to find morphine in human saliva was an unexpected result, since the saliva test is widely used in horse racing. In 1893 Rosenwald (8) reported traces of morphine in the saliva of a man after the injection of therapeutic doses, and he considered the morphine found in the stomach to have been swallowed with the saliva. He was unable to determine the rate of excretion in saliva.

No satisfactory explanation can be offered as to why no morphine has been found in blood of morphine addicts. If morphine is present the concentration must be low (less than 0.10 mgm. per 100 grams). Plant and Pierce (7) found 0.40 to 1.29 mgm. per 100 grams blood in tolerant dogs, the daily dosage range being 300 to 700 mgm. morphine sulphate, or approximately 50 mgm. per kilogram of body weight. The daily dosage range studied in man was

from 200 to 600 mgm., which is approximately 3 to 9 mgm. per kilogram of body weight. The lower dosage level may be one reason why morphine has not been found in the blood of morphine addicts. Another possibility is that morphine may adhere to protein and be precipitated, or may resist solution when the sample is prepared for extraction. In the tolerant and non-tolerant animals studied by Plant and Pierce the morphine concentration of blood was barely perceptible by their method.

The morphine found in liver only indicates that the morphine-molybdate-vanadate method is sufficiently sensitive to detect its presence. It was shown by Plant and Pierce in both the tolerant and non-tolerant dogs that liver was the tissue containing the highest concentration of morphine. It is regrettable that tests were not made here for bound morphine, for there is reason to believe that the site of morphine conjugation is in the liver. The finding of only bound morphine in bile strengthens this hypothesis.

SUMMARY

A modified method for the micro-determination of morphine in quantities from 0.03 to 0.2 mgm. by the morphine-molybdate-vanadate procedure has been described. Morphine analyses were made on urine, feces, saliva, gastric contents, perspiration, bile, and blood from morphine addicts, as well as liver obtained at autopsy on two patients who were addicted at the time of death.

Feces, liver, and perspiration contained free morphine; bile contained only bound morphine; urine and gastric contents contained both free and bound morphine; no morphine was found in saliva or blood.

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A THERAPEUTIC INCOMPATIBILITY BETWEEN SULFAPYRIDINE AND QUININE¹

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During the course of some experiments designed to study the factors controlling the production and excretion of acetylsulfapyridine we made the unexpected observation that quinine administered to rats receiving sulfapyridine markedly increased the total amount and the concentration of acetylsulfapyridine² in the urine. It is well recognized that, in man and in several species of experimental animals, some of the most objectionable aspects of the toxicity of sulfapyridine result from the precipitation of crystals of acetylsulfapyridine in the kidney and urinary passages (2-9); hence an investigation of this tendency of quinine to exaggerate the incidence of urinary obstruction would appear important. The purpose of this paper is to present some data on the quantitative changes in the urinary excretion of acetylsulfapyridine produced by quinine.

MATERIALS AND METHODS. *Animals.* Excluding the preliminary experiments, 108 male, white rats from the Wistar strain were used. The animals were arranged in two groups according to age, one ranging from 56 to 89 days, and the other from 146 to 218 days. The parallelism between the reactions of the two groups was very close and the divergencies involved only quantitative differences, favoring a greater toxicity in the older animals. Individual metabolic cages providing free access to water and food were used. The diet consisted of a mixture of 93 per cent ground Purina dog chow checkers and 7 per cent lard. The latter was added to diminish scattering. The urine was collected in periods of 24 hours \pm 30 minutes and each period ended immediately before the morning administration of the drugs. Since the time between the final dose of sulfapyridine and the end of a period for the collection of urine was 12 or 16 hours, depending upon the daily dose of sulfapyridine, the small error in the time of the collection became insignificant.

Drugs. Powdered sulfapyridine suspended in a 2 per cent solution of starch, with or without quinine bisulfate, was administered in equal doses by stomach tube between

¹ This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

² The terms acetylsulfapyridine and combined sulfapyridine have been used interchangeably to designate the increase in the values for sulfapyridine obtained after the hydrolysis by acid described in the method of Bratton and Marshall (1).

9 and 10 a.m., and 4 and 5 p.m. The daily dosages per kilogram were: sulfapyridine, 120 mgm.; quinine bisulfate, 30, 40, 60, 120 or 240 mgm.; and solution of starch, 24 cc. In a few experiments the daily dosages of sulfapyridine and starch were doubled and administered in equal quantities at 11 a.m., 3, 5, and 9.30 p.m. In some experiments atabrine, obtained from atabrine tablets, was substituted for the quinine. Determinations of the free and acetylated sulfapyridine were made by the method of Bratton and Marshall (1); a photo-electric colorimeter was used. Neither quinine nor its metabolic products interfered with this method in the dilutions employed in our work.

RESULTS. *Effect of quinine on the total quantity of sulfapyridine in the urine.* The data in table 1 show that the urinary excretion of free and combined sulfapyridine was remarkably constant over a period of 4 days when the rats were given 120 mgm. per kilogram of sulfapyridine daily. In the younger rats, ranging in age from 60 to 89 days, and in weight from 168 to 268 grams, receiving no quinine, the average excretion of total sulfapyridine in milligrams per kilogram per day varied less than 5 per cent. In the corresponding group of older rats, ranging in age from 172 to 218 days, and in weight from 343 to 473 grams, the daily excretion after the first 24 hours was equally as constant as that of the younger rats. The values for the standard error of the mean calculated for the excretion of the free and combined drug (table 1) show that there was a satisfactory consistency in the excretions from the individual animals.

Quinine bisulfate administered with the sulfapyridine increased the excretion of total sulfapyridine in proportion to the dose of quinine for the range investigated (fig. 1). On the first day this effect was small but distinguishable. However, on the second, third and fourth days the increased excretion was marked, averaging in the younger rats for these days from 13.5 to 43.6 per cent for a dosage of 30 to 240 mgm. per kilogram per day, respectively, of quinine bisulfate. In the absence of quinine the younger rats excreted the following percentages of the daily dose of sulfapyridine: first day, 53.6; second day, 52.7; third day, 53.2; fourth day, 51.0. Analogous data following the administration of 240 mgm. per kilogram per day of quinine bisulfate with the sulfapyridine were: first day 54.2; second day, 67.3; third day, 81.5; fourth day, 77.0 (table 1).

The values for the total sulfapyridine in the blood support the urinary data in indicating a more complete absorption of sulfapyridine in the presence of quinine. Analyses on whole blood drawn from the older rats on the second day of the administration of the drugs, 4 hours after the morning dosage, using 120 mgm. per kilogram per day of sulfapyridine, with and without quinine, gave the following average values for the total sulfapyridine: no quinine, 3.2 mgm. per cent; 120 mgm. per kilogram per day of quinine bisulfate, 3.9 mgm. per cent; and 240 mgm. per kilogram per day of quinine bisulfate, 4.9 mgm. per cent. In a corresponding experiment, except for the omission of the larger dose of quinine, the average total sulfapyridine values for blood drawn 5 hours after the morning dosage of the drugs were: no

TABLE 3 1
*Effects of quinine on the urinary excretion of sulfapyridine**

NUM- BER OF AN- THRAL MALE	AGE RANGE	DAILY DOSAGE OF QUININE BISULFATE	SULFAPYRIDINE EXCRETED															
			First day		Second day				Third day				Fourth day				Average daily ex- cretion—second, third, and fourth days	
			Free	Com- bined	Free S.E.M	Com- bined	S.E.M	Free S.E.M	Com- bined	S.E.M	Free S.E.M	Com- bined	S.E.M	Free	Com- bined			
			mgm. /kgm.	mgm. /kgm.	mgm. /kgm.	mgm. /kgm.	±	mgm. /kgm.	mgm. /kgm.	±	mgm. /kgm.	mgm. /kgm.	±	mgm. /kgm.	mgm. /kgm.	±	mgm. /kgm.	mgm. /kgm.
11	60-89	0	31.2	33.1	29.3	1.69	34.1	2.12	27.0	2.71	36.8	2.42	26.9§	2.16	34.3§	1.95	27.7	35.1
5	60-71	30	34.8	38.7	30.2	2.52	40.0	2.47	29.9	3.51	40.3	2.05	27.8	2.08	43.5	2.08	30.0	41.3
5	60-65	40	33.7	33.9	27.3	2.23	34.3	2.17	28.4	2.08	39.5	1.50	32.9	1.62	48.2	0.56	29.5	40.7
4	56-60	60	31.4	37.6	28.9	3.48	40.1	1.44	31.3	4.51	42.7	3.14	31.0	2.08	50.1	3.03	30.4	44.2
5	56-66	120	29.4	44.4	29.4	1.00	55.3	3.27	27.9	1.12	63.5	7.10	25.5	1.30	55.1	2.72	27.6	57.6
5	56-62	240	18.9	46.2	20.4	1.34	60.3	1.76	21.2	1.65	76.5	3.28	22.7	1.26	69.7	3.27	21.4	68.8
8	172-218	0	25.7	30.6	28.2	1.81	36.9	1.23	27.5	2.08	38.1	1.79	27.5	2.14	40.1	2.05	27.7	38.4
5	175-176	30	24.4	37.6	24.8	1.55	45.3	2.12	24.7	0.84	50.0	1.60	24.5	1.68	47.3	3.37	24.7	47.3
5	174-175	60	19.6	39.1	18.2	0.91	45.6	3.66	23.2	2.16	54.4	3.76	17.7	3.07	50.2	9.23	19.7	50.1
													20.7**		59.1**			
7	113-118	25.9	22.0	31.5	20.1	0.17	35.3	0.60	19.1	2.97	36.3	1.13	22.7	0.98	40.3	1.68	20.6	37.3
7	116-118	72.5	15.0	36.1	16.7	0.80	55.6	1.67	18.0	3.41	56.2	2.72	21.2	1.69	61.8	3.65	18.6	57.9

* The dosage of sulfapyridine was 120 mgm./kgm./day.

† Sulfate content equivalent to that of 120 mgm. of quinine bisulfate.

‡ Quinine content equivalent to that of 120 mgm. of quinine bisulfate.

§ Average for 5 rats.

** Average for 4 rats. One rat in this series gave on the fourth day an abnormally small volume of urine with a proportionately small excretion of sulfapyridine, probably the result of a congested kidney. For this reason averages with and without this rat have been recorded.

quinine, 2.8 mgm. per cent; 120 mgm. per kilogram per day of quinine bisulfate, 3.1 mgm. per cent.

Effect of quinine on the relation of free to acetylated sulfapyridine in the urine. In figure 1 we have plotted the average daily excretion of free, acetylated, and total sulfapyridine for 4 consecutive days from rats given sulfapyridine, alone and with graded quantities of quinine bisulfate. The increased absorption and excretion of sulfapyridine in the presence of quinine might have some therapeutic advantage were it not for the fact that the percentage of sulfapyridine excreted in the acetylated form increased to such a degree that the

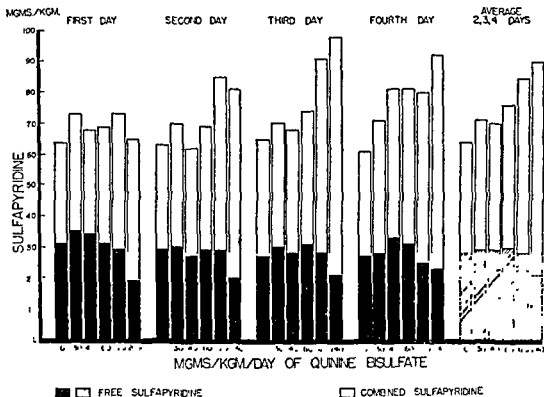


FIG. 1. EFFECTS OF QUININE BISULFATE ON THE URINARY EXCRETION OF SULFAPYRIDINE

Dosage of sulfapyridine was 120 mgm./kgm./day. Thirty-five rats ranging in age from 56 to 89 days were used.

total amount of sulfapyridine in the free state either remained essentially unchanged or decreased (fig. 1). The averages for the last 3 days of the experiment show that 30 mgm. per kilogram per day of quinine bisulfate increased the amount of acetylsulfapyridine excreted in the urine 18 per cent; 120 mgm. increased it 64 per cent, and 240 mgm., 96 per cent. A more complete analysis of these changes is recorded in table 2. This increase in the acetylsulfapyridine would be less objectionable if there were a corresponding increase in the volume of the urine to insure its solution, but the volume of the urine per unit of body weight in those rats receiving quinine and sulfapyridine was the same as for those receiving only sulfapyridine.

For the range of drugs employed in our experiments the percentage of sulfapyridine excreted free bears a linear relationship to the dose of quinine in the group of younger animals (fig. 2). Since, with a constant dosage, the percentage of sulfapyridine excreted free changed slightly from the second through the fourth day of our experiments, it appeared that a more accurate evaluation of the effect of quinine would be obtained by selecting the data for a single day. The second experimental day was chosen because it provided the maximal amount of data. When the daily dosage of sulfapyridine was 120 mgm. per kilogram, the average value for the percentage of sulfapyridine excreted free in the second day's urine decreased from 46 in the ab-

TABLE 2
Effects of quinine on the urinary excretion of sulfapyridine

NUMBER OF ANIMALS	AGE RANGE	DRUGS AND DAILY DOSAGE		EXCRETED SULFAPYRIDINE INGESTED SULFAPYRIDINE		CHANGE IN EXCRETION IN PRESENCE OF QUININE†				SULFAPYRIDINE EXCRETED FREE TOTAL SULFAPYRIDINE EXCRETED	
		Quinine bisulfate	Sulfapyridine	Second day	Third day	Free sulfapyridine		Acetylsulfapyridine		Second day	Third day
						Second day	Third day	Second day	Third day		
	days	mgm. per kgm.	mgm. per kgm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
11	60-89	0	120	52.7	53.2					46.4	42.6
5	60-71	30	120	58.5	58.5	+3.1	+10.7	+17.3	+9.5	43.0	42.6
4	56-60	60	120	57.5	61.7	-1.4	+15.9	+17.3	+16.0	41.9	42.4
5	56-66	120	120	70.7	76.0	+0.3	+3.3	+62.1	+72.5	34.7	30.5
5	56-62	240	120	67.3	81.5	-30.4	-21.5	+76.9	+108.0	25.2	21.7
4	85-86	0	240*†	57.5	52.5					40.0	42.0
4	64-82	120	240*	67.5	73.4	+6.71	+11.7	+24.9	+59.6	36.1	33.8

* Drugs administered at 11 a.m., 3, 5, and 9:30 p.m.

† The additional water given by stomach tube did not change the volume of the urine.

‡ All calculations were based on milligrams of drug per kilogram of body weight.

sence of quinine, to 25 with a daily dose of 240 mgm. of quinine bisulfate per kilogram (fig. 2). In the group of older rats, also shown in figure 2, quinine at each level of dosage produced a greater depression in the percentage of sulfapyridine excreted free. The greatest divergence between the groups occurred in the quinine bisulfate range of 60 to 120 mgm. per kilogram per day. Here the percentages of free sulfapyridine in the urine were 42 and 35, respectively, for the younger rats, but 29 and 23, respectively, for the older animals. When one corrects these values for the difference, between the groups, in the percentages of sulfapyridine excreted free in the absence of quinine, the values are not changed appreciably. It is also apparent from figure 2 that the relation, between the dosage of quinine and the percentage of sulfa-

pyridine excreted free in the urine, is not the same in the younger and older animals. In the older rats the relation appears to be logarithmic. In figure 3 the percentage of free sulfapyridine excreted by the older rats has been plotted against the dosages of quinine bisulfate on a logarithmic scale with the result that a straight line relationship has been demonstrated.

In a few experiments we attempted to determine the limits of the action of quinine; accordingly we increased the daily dosage of sulfapyridine from 120 to 240 mgm. per kilogram. With the larger dosage of sulfapyridine the daily dosage of quinine bisulfate was 120 mgm. per kilogram. The results in

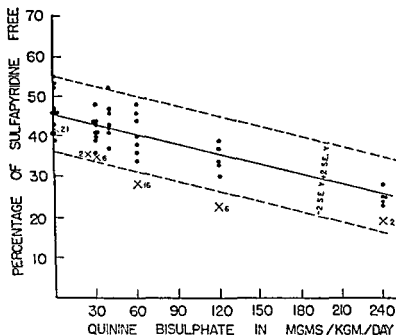


FIG. 2. EFFECTS OF QUININE ON THE PERCENTAGE OF SULFAPYRIDINE EXCRETED FREE

Dosage of sulfapyridine was 120 mgm./kgm./day. ●, values for second day of experiment, individual rats, 56 to 89 days of age. X, average values for second day of experiment, rats ranging in age from 172 to 218 days. The numeral adjacent to X represents the number of rats.

table 2 show that in the absence of quinine the ratio of excreted to ingested sulfapyridine is the same for a dosage of 240 mgm. as for 120 mgm. Also, this table shows that in the presence of a daily dosage of 120 mgm. of quinine bisulfate the ratio of excreted to ingested sulfapyridine is essentially the same for the dosage of 240 mgm. of sulfapyridine as for that of 120 mgm. This ratio is smaller than the one obtained when the dosage of quinine is 240 and that of sulfapyridine 120, but larger than the one obtained when the dosage of quinine is 60 and that of sulfapyridine 120. With a daily dosage of 120 mgm. of quinine bisulfate per kilogram the percentage of sulfapyridine excreted free was slightly greater with the larger dosage of sulfapyridine and the increase in the excretion of acetylsulfapyridine expressed in per cent was

smaller with the larger dosage (table 2). However, the absolute increase in the excretion of acetylsulfapyridine effected by the 120 mgm. dosage of quinine bisulfate on the second experimental day was 20.5 mgm. per kilogram for the 240 mgm. dosage of sulfapyridine and 21.2 mgm. per kilogram for the smaller dosage of sulfapyridine. Corresponding values for the third day were 43.5 and 26.7.

Although it appeared highly improbable that these effects could be at-

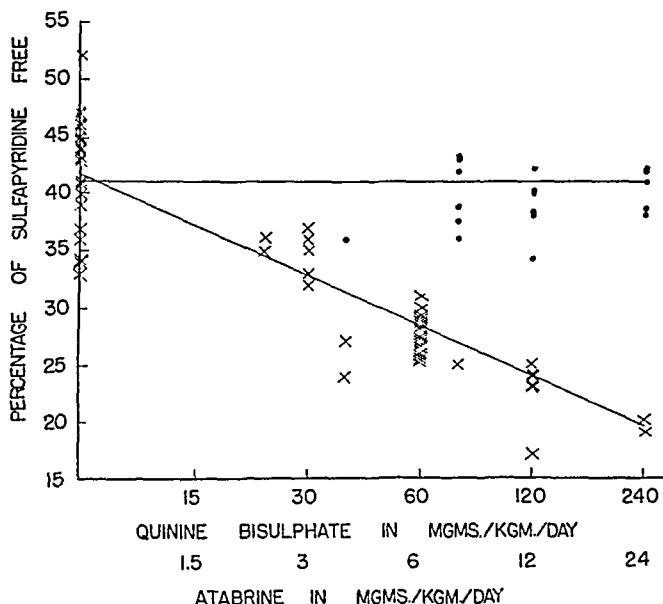


FIG. 3. EFFECTS OF QUININE AND ATABRINE ON THE PERCENTAGE OF SULFAPYRIDINE EXCRETED FREE

Dosage of sulfapyridine was 120 mgm./kgm./day. X, values for quinine bisulfate on second day of experiment, individual rats, 140 to 218 days of age. ●, values for atabrine on second day of experiment, individual rats 146 to 218 days of age.

tributed to any factor other than the quinine, it seemed advisable to exclude the possibility that the results might be due to the sulfate in the quinine salt. Accordingly, in one group of rats the quinine bisulfate was replaced by sodium sulfate, and in another group by quinine hydrochloride. The sulfate and the quinine in these substitutes were equivalent stoichiometrically to the sulfate and quinine content of 120 mgm. of quinine bisulfate. The results, shown in table 1, demonstrate that sodium sulfate did not increase the ratio of excreted to ingested sulfapyridine or the amount of acetylsulfapyridine excreted. However, it depressed somewhat the amount of free

sulfapyridine in the urine. Quinine hydrochloride duplicated satisfactorily the action of quinine bisulfate.

The changes in the excretion of sulfapyridine produced by quinine are temporary and disappear rapidly when quinine is withdrawn. The rate of the disappearance apparently parallels the excretion of the quinine. In a group of the older rats given 120 mgm. of sulfapyridine per kilogram daily with an equal weight of quinine bisulfate for three days, the percentage of sulfapyridine excreted free was 31.4 on the first day, 23.4 on the second day, and 23.3 on the third day. No drugs were administered on the fourth and

TABLE 3
*Effect of atabrine on the urinary excretion of sulfapyridine**

NUMBER OF ANIMALS	AGE RANGE	DAILY DOSAGE OF ATABRINE	EXCRETED SULFAPYRIDINE INGESTED SULFAPYRIDINE			CHANGE IN EXCRETION OF ACETYSULFAPYRIDINE IN PRESENCE OF ATABRINE†			SULFAPYRIDINE EXCRETED FREE TOTAL SULFAPYRI- DINE EXCRETED
			Second day	Third day	Fourth day	Second day	Third day	Fourth day	Average—second, third, and fourth days
	days	mgm. per kgm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
8	172-218	0	51.3	51.6	56.5				41.9
2	201-202	4	51.5	55.2	52.4	-1.1	+1.8	-14.2	42.6
6	172-202	8	51.2	49.9	58.5	-0.8	-4.5	+2.5	40.4
6	159-188	12	54.8	57.7	56.1	+8.9	+9.5	+1.3	39.2
5	146-166	24	50.6	60.6	56.6	-2.4	+15.7	+3.2	39.7
2	350-368	48†	45.4			-12.3			38.3
2	559-560	96†	53.2			-5.5			43.4
2	528-529	176†	49.0			-8.9			40.6

* The dosage of sulfapyridine was 120 mgm./kgm./day.

† Atabrine administered on first day only.

‡ All calculations were based on milligrams of drug per kilogram of body weight.

fifth days and the combined urines for these days showed that the percentage of sulfapyridine excreted free had risen to 38.1. Corresponding values in the controls administered no quinine were: first day 45.1, second day 42.1, third day 43.1, fourth and fifth days 46.4.

Atabrine. An examination of the action of atabrine to determine whether or not this drug resembled quinine in its effect upon the excretion of sulfapyridine appeared to have some therapeutic interest. Since atabrine is used in the treatment of malaria in approximately one-tenth of the weight employed for the salts of quinine this ratio was adhered to in some of our experiments in which atabrine was substituted for the quinine bisulfate. The data in figure 3 and table 3 show that atabrine does not appreciably influence the excretion of sulfapyridine.

DISCUSSION. Using a daily dosage of 1 gram of sulfapyridine per kilogram, under conditions somewhat different from those of our experiments, Scudi and Robinson (10) observed that 3 or 4 days were required to attain a constant urinary excretion of the free drug, although the excretion of acetylsulfapyridine became constant in 24 hours. In our experiments the excretion of both of these products became constant in 24 to 48 hours. We believe that the apparent discrepancy between the results from the two laboratories is attributable to the large difference between the dosages employed.

The increased absorption of sulfapyridine in the presence of quinine corresponds to the results reported by Hanzlik and Cutting (11) and also by Driver and Murlin for insulin and quinine (12). These authors observed that quinine facilitated the absorption of insulin from the intestine of man, rabbits and dogs by a mechanism independent of its antienzymatic action.

Similar analogies for the action of quinine on the conjugation of sulfapyridine have not been found. Quinine inhibits the conjugation of glycine and benzoic acid in the perfused kidney (13), oxidations in the excised muscle (14), and autolysis in the excised liver (15). These observations suggested that it might inhibit the acetylation of sulfapyridine and thereby enhance this sulfonamide's therapeutic value and decrease its toxicity. The disclosure of an effect the opposite from that expected suggests at least two explanations: (a) that quinine exerts a greater inhibition on the system responsible for the deacetylation of sulfapyridine than on that responsible for the acetylation (16); (b) that it increases the differential between the renal clearance of free and acetylated sulfapyridine (17). In support of the first suggestion, the average values for the percentage of combined sulfapyridine in the blood are slightly though consistently higher in the rats receiving quinine. On a daily dosage of 120 mgm. of sulfapyridine per kilogram the average percentages of combined sulfapyridine in the blood of the older rats, 4 hours after the morning administration of the drugs on the second day of the experiment were: without quinine, 51 per cent; with 120 mgm. of quinine bisulfate per kilogram per day, 56 per cent; with a daily dose of 240 mgm. of quinine bisulfate per kilogram, 59 per cent. On the fourth day of a parallel experiment, in which only the smaller dose of quinine was used, 5 hours after the morning dosage the average values were: without quinine, 40 per cent combined; with quinine, 45 per cent combined. These results do not exclude the possibility of a renal action but additional experimental data will be required to support the suggestion.

In view of the close similarity between the conjugation and excretion of sulfapyridine in man and the rat it appears that treatment in the human of a double infection for which quinine and sulfapyridine would be simultaneously used should be managed by other drugs until it is established that the quantities of these drugs employed in human therapy do not increase the hazard of urolithiasis.

SUMMARY

1. Quinine administered orally with sulfapyridine to rats increased the total amount of sulfapyridine absorbed and excreted in the urine. The maximal increase was produced by the largest dose of quinine and, using average values, amounted to 44 per cent.

2. The increase in the urinary excretion of total sulfapyridine represented principally an increase in the acetylsulfapyridine. The largest dose of quinine produced an average increase of 96 per cent in the excretion of acetylsulfapyridine. This effect was not accompanied by an increase in the volume of urine.

3. Quinine depressed the free sulfapyridine in the urine. The maximal decrease observed was 33 per cent.

4. Atabrine substituted for quinine produced no comparable effect.

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AN IN VITRO STUDY ON THE SYNERGISTIC ACTION OF SULFAMIDO COMPOUNDS AND AZOCHLORAMID UPON VARIOUS PATHOGENIC MICROORGANISMS

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It is generally agreed that sulfanilamide and its derivatives are highly effective in the treatment of diseases caused by a variety of bacteria, a few viruses and some parasites. On the other hand, the results obtained with these drugs are frequently less satisfactory in localized purulent lesions and in certain subacute and chronic infections. Failure of chemotherapy in these conditions may be due in part either to drug-fastness of the microorganisms or to the presence of anti-sulfanilamide factors such as peptone, *p*-amino-benzoic acid and certain growth factors in inflammatory exudates.

It has been shown that the antibacterial activity of sulfamido compounds can be markedly enhanced by the addition of immune serums. In an extensive study on drug and serum therapy of experimental meningococcal infections of mice, Branham (1) showed that the combination of sulfanilamide or sulfapyridine with meningococcal antiserum gave results far better than with either agent alone. Moreover, she made the interesting observation that a serum that had practically no protective action when used alone gave marked protection when it was given together with the sulfamido compounds. Likewise, there is some clinical evidence that the administration of both sulfamido compounds and anti-pneumococcal serum may yield better results than treatment with either alone. The question arises as to whether or not similar synergistic effects may be obtained by the combined use of sulfamido compounds and other specific chemotherapeutic agents or ordinary disinfectants. Osgood and his co-workers (2) studied the effect of neoarsphenamine plus sulfathiazol in marrow cultures and suggested the combined use of these drugs in the treatment of severe infections of man. Zaytzeff-Jern and Meloney (3) reported favorable results in the treatment of staphylococcal infections by the combined use of sulfamido compounds and specific bacteriophage. Sulfamido compounds not only somewhat increased the lytic activity of bacteriophage, but also inhibited the development and growth of phage-resistant staphylococci (4).

In vitro experiments on the combined effectiveness of two chemotherapeutic

substances active against pneumococci failed to reveal such a synergistic action: Sulfapyridine and sulfathiazol used together with optochin hydrochloride (ethylhydrocupreine hydrochloride) or β -hydroxyethylapocupreine dihydrochloride (Parke, Davis & Co.) exerted no increased bacteriostatic activity beyond that of either compound in like concentration alone (5). Likewise, no evidence of a synergistic action was obtained when sulfamido compounds were combined with certain ordinary disinfectants, e.g., merthiolate. Nor did any increased bacteriostatic effectiveness toward Group A hemolytic streptococcus result from the combined use of sulfanilamide and actinomycin A, obtained through the courtesy of Dr. Selman A. Waksman, New Jersey Agricultural Experimental Station, New Brunswick. On the other hand, it could be shown (6) that the combined use of pyridium (phenylazo- α - α -diaminopyridine mono-hydrochloride) and sulfamido compounds exerted greater bacteriostatic activity toward *B. coli* *in vitro* than either compound alone: 5 mgm. per cent of sulfapyridine combined with pyridium in a dilution of 1:6,000 completely prevented growth of the organisms, whereas sulfapyridine alone, even in a concentration of 50 mgm. per cent, and pyridium alone (1:6,000) failed to do so. In a preliminary note (7) it was reported that sulfanilamide and azochloramid in combination produced greater bacteriostatic effects toward hemolytic streptococcus than either agent alone. Azochloramid (*N, N'*-Dichloroazodicarboxamidine (Wallace & Tiernan Products)) was chosen as a relatively stable bacteriostatic and bactericidal chlorine compound whose effectiveness in the presence of organic matter is inhibited to a slight degree only.

The results of *in vitro* experiments on the synergistic action of sulfamido compounds (such as sulfanilamide, sulfapyridine, sulfathiazol and sulfadiazine) and azochloramid upon various pathogenic microorganisms are presented in this communication.

MATERIAL AND METHODS. The strains of microorganisms used in these studies were isolated from human sources. Group A hemolytic streptococcus was recovered from empyema fluid; Group D hemolytic streptococcus was obtained from the urine of a patient with pyelitis; *staphylococcus aureus hemolyticus* was recovered from the empyema fluid of a child; and pneumococcus type I from the sputum of a patient with pneumonia. All the strains were kept on blood agar plates prepared with human blood. The strain of staphylococcus was pathogenic according to the plasma coagulase test. The strain of pneumococcus type I even in high dilution killed both mice and rabbits.

As culture medium brain heart infusion (Difco) (containing infusion from calf brains and beef heart, 1 per cent proteose peptone, 0.2 per cent dextrose, 0.5 per cent sodium chloride and 0.25 per cent disodium phosphate) was employed. The pH of the broth was 7.4. The broth was sterilized by autoclaving at 15 pounds pressure for 15 minutes.

The following sulfamido compounds were used in these studies: sulfanilamide, (*p*-aminobenzenesulfonamide, Winthrop); sulfapyridine (2-sulfanilyl aminopyridine, Merck); sulfathiazol (2-sulfanilamidothiazol, Winthrop); and sulfadiazine (2-sulfanilamidopyrimidine, Lederle). These drugs were dissolved in appropriate amounts in infusion broth and the broths were sterilized by autoclaving at 15 pounds pressure for 15 minutes.

Azochloramid (*N,N'*-Dichloroazodicarboxamidine, Wallace & Tiernan Products) was dissolved in sterile broth and kept in the dark. The azochloramid broth was not heated; controls for sterility were carried out.

The bacteriostatic activity of these drugs, alone and in combination, was estimated on the basis of inhibition of visible growth. In some experiments the number of viable organisms was determined by means of poured blood agar plates. The specimens were diluted in broth containing *p*-aminobenzoic acid and/or sodium sulfite in order to counteract the activity of sulfanilamide and azochloramid.

RESULTS. *Experiment I: The combined bacteriostatic activity of azochloramid and sulfathiazol, alone and in combination, upon Group A hemolytic streptococcus.* In this experiment sulfathiazol was used in concentrations ranging from 0.1 to 100 mgm. per cent and azochloramid in concentrations of 1:100,000 and 1:150,000. When used in combination sulfathiazol was studied in concentrations from 0.1 to 100 mgm. per cent and azochloramid in a dilution of

TABLE 1

Bacteriostatic activity of azochloramid and sulfathiazol, alone and in combination, upon group A hemolytic streptococcus in infusion broth

HOURS OF INCUBATION AT 37° C.	SULFATHIAZOL (MG. PER CENT)					SULFATHIAZOL, MG. PER CENT AND AZOCHLORAMID 1:200,000					AZOCHLORAMID	
	0	0.1	1	10	100	0	0.1	1	10	100	1:150,000	1:100,000
1) 18	++++	+++	++++	++++	-	+	-	-	-	-	-	-
2) 48	++++	++++	++++	++++	-	++++	++++	-	-	-	-	-
3) 72	++++	++++	++++	++++	-	++++	++++	-	-	-	++++	-
4) 96	++++	++++	++++	++++	-	++++	++++	-	-	-	++++	++++
5) 120	++++	++++	++++	++++	-	++++	++++	-	-	-	++++	++++

-, no visible growth; + to +++++, various degrees of visible growth.

1:200,000. Broth without any drugs was used as a control. The broths (volume 4.8 cc.) were seeded with 0.2 cc. of a 1:5 diluted 18 hours infusion broth culture of Group A hemolytic streptococcus. The tubes were kept at 37°C. in the dark. The resulting growth was noted at various intervals. The results of this experiment are presented in table 1.

It may be seen from table 1 that sulfathiazol in combination with azochloramid exerted greater bacteriostatic activity upon this strain of Group A hemolytic streptococcus than either drug alone. When used together with azochloramid (1:200,000) growth was completely and continuously inhibited by sulfathiazol in a concentration of 1 mgm. per cent. In contrast, sulfathiazol alone in a concentration of 10 mgm. per cent as well as azochloramid alone in a concentration of 1:100,000 failed to prevent the growth of the organism. Thus azochloramid and sulfathiazol in combination exerted greater bacteriostatic activity than ten times the concentration of sulfathiazol alone or twice the concentration of azochloramid alone.

Experiment II: The combined bacteriostatic activity of azochloramid and

sulfadiazine, alone and in combination, upon Group A hemolytic streptococcus. Recent experimental and clinical investigations have shown the antibacterial efficacy of another sulfanilamide derivative, namely, *sulfadiazine*. It was thought desirable, therefore, to study the anti-streptococcal activity of *sulfadiazine* and *azochloramid*, alone and in combination. A representative experiment was as follows: Infusion broth (volume 4.8 cc.) containing (1) *sulfadiazine* in concentrations ranging from 0.1 to 100 mgm. per cent; (2) *azochloramid* in concentration of 1:150,000; (3) *azochloramid* (1:200,000) together with *sulfadiazine* (0.1 to 100 mgm. per cent) was inoculated with 0.2 cc. of a 1:5 diluted 18 hours infusion broth culture of Group A hemolytic streptococcus. The tubes were incubated for 4 days at 37°C. The results of this experiment are presented in table 2, which shows that *sulfadiazine* alone even in concentrations up to 100 mgm. per cent failed to prevent the growth of the streptococcus. Likewise, *azochloramid* in a concentration of 1:150,000 did not

TABLE 2

Bacteriostatic activity of azochloramid and sulfadiazine, alone and in combination, upon group A hemolytic streptococcus in infusion broth

HOURS OF INCUBATION AT 37°C.	SULFADIAZINE (MG. PER CENT)					SULFADIAZINE, MG. PER CENT AND AZOCHLORAMID 1:200,000					AZOCHLORAMID 1:150,000
	0	0.1	1	10	100	0	0.1	1	10	100	
1) 18	++++	++++	++++	++++	+++	++++	++++	++++	-	-	++++
2) 48	++++	++++	++++	++++	+++	++++	++++	++++	-	-	++++
3) 72	++++	++++	++++	++++	+++	++++	++++	++++	-	-	++++
4) 96	++++	++++	++++	++++	+++	++++	++++	++++	-	-	++++

—, no visible growth; + to +++++, various degrees of visible growth.

inhibit its growth. On the other hand, *sulfadiazine* in concentration of 10 mgm. per cent used in conjunction with *azochloramid* in a concentration of 1:200,000 completely and continuously inhibited the growth of the streptococcus. As previously reported, a similar synergistic action toward Group A hemolytic streptococcus was obtained by the combined use of *azochloramid* and sulfanilamide. It may be concluded from these experiments, therefore, that the combined use of *azochloramid* and sulfamido compounds exerts greater anti-streptococcal activity than does either compound alone in like or somewhat higher concentrations. A quantitative study confirmed these results. In one representative experiment the numbers of viable streptococci per 0.2 cc. after 24 hours' incubation at 37°C. were as follows: (1) Control broth: 4,000,000; (2) 100 mgm. per cent sulfanilamide broth: 2,000,000; (3) 1:800,000 *azochloramid* broth: 3,000,000; (4) 1:200,000 *azochloramid* broth: 600; (5) broth containing 25 mgm. per cent of sulfanilamide and *azochloramid* in a dilution of 1:800,000: 2,100. In the same experiment, after 48 hours' incubation, no viable streptococci were demonstrable in broth con-

taining 25 mgm. per cent of sulfanilamide and azochloramid in a dilution of 1:400,000, whereas broth containing azochloramid alone in a dilution of 1:200,000 had 8,000,000 viable streptococci.

Experiment III: The combined bacteriostatic activity of azochloramid and sulfanilamide, alone and in combination, upon Group D hemolytic streptococcus. The bacteriostatic activity of sulfanilamide in concentrations of 10 to 1000 mgm. per cent and of sulfanilamide in combination with azochloramid in concentrations of 1:100,000 and 1:50,000, respectively, toward Group D hemolytic streptococcus was investigated. For comparison, azochloramid alone in a concentration of 1:20,000 was included. The broths (volume 4.8 cc.) were seeded with 0.2 cc. of a 1:1,000,000 diluted 18 hours infusion broth culture of the streptococcus. The tubes were incubated at 37°C. for 6 days and the resulting growth was recorded at various intervals. Table 3 gives the results of this experiment.

TABLE 3

Bacteriostatic activity of azochloramid and sulfanilamide, alone and in combination, upon group D hemolytic streptococcus in infusion broth

HOURS OF INCUBA- TION AT 37°C.	SULFANILAMIDE (MGM. PER CENT)				SULFANILAMIDE, MG. PER CENT AND AZOCHLORAMID 1:100,000				SULFANILAMIDE, MG. PER CENT AND AZO- CHLORAMID 1:50,000				AZO- CHLOR- AMID 1:20,000
	0	10	100	1000	0	10	100	1000	0	10	100	1000	
1) 18	++++	++++	++++	-	++++	++	-	-	-	-	-	-	-
2) 48	++++	++++	++++	+++	++++	++++	++++	-	++++	-	-	-	-
3) 72	++++	++++	++++	++++	++++	++++	++++	++++	++++	-	-	-	-
4) 96	++++	++++	++++	++++	++++	++++	++++	++++	++++	-	-	-	-
5) 120	++++	++++	++++	++++	++++	++++	++++	++++	++++	-	-	-	-
6) 144	++++	++++	++++	++++	++++	++++	++++	++++	++++	-	-	-	-

-, no visible growth; + to +++++, various degrees of visible growth.

It may be seen from table 3 that sulfanilamide in concentrations up to 100 mgm. per cent failed to exert any bacteriostatic activity toward this strain of Group D hemolytic streptococcus; in concentration of 1000 mgm. per cent it delayed the growth of the organism, but did not completely prevent it. Azochloramid alone in concentration of 1:20,000 inhibited the growth of the streptococcus; in concentrations of 1:50,000 and less its bacteriostatic activity was slight. The table, furthermore, shows that the combined use of both drugs was slightly more effective than that of either drug in like concentration alone. In combination, sulfanilamide in concentration of 100 mgm. per cent and azochloramid in concentration of 1:100,000 delayed the growth of the organism to approximately the same extent as did sulfanilamide alone in a concentration of 1000 mgm. per cent and azochloramid alone in a concentration of 1:50,000. It is interesting to note that sulfanilamide in a concentration of 10 mgm. per cent used in conjunction with azochloramid (1:50,000) exerted greater growth-inhibiting activity than sulfanilamide alone

in a concentration of 1000 mgm. per cent. However, this combination of the two drugs was not more effective than azochloramid alone in concentration of 1:20,000. Similar results were obtained in several experiments, indicating that the combined use of azochloramid and sulfanilamide is only slightly more effective toward Group D hemolytic streptococcus than either compound alone.

Experiment IV: The combined bacteriostatic activity of azochloramid and sulfadiazine, alone and in combination, upon pneumococcus type I. Infusion broth containing (1) sulfadiazine in concentrations ranging from 0.1 to 100 mgm. per cent; (2) azochloramid (1:150,000); (3) sulfadiazine (0.1 to 100 mgm. per cent) plus azochloramid in concentrations of 1:200,000 and 1:400,000, respectively, was inoculated with 0.2 cc. of a 1:5 diluted 18 hours broth culture of pneumococcus type I. The tubes were incubated at 37°C. for 6 days and the growth was noted at various intervals. The results of this experiment are presented in table 4.

Table 4 reveals that sulfadiazine even in concentrations up to 100 mgm. per cent and azochloramid in a concentration of 1:150,000 failed to prevent the growth of the pneumococcus, although azochloramid definitely delayed it. In contrast, sulfadiazine in concentration of 10 mgm. per cent and above used together with azochloramid in a concentration of 1:200,000 caused complete bacteriostasis over the entire period of observation. Essentially the same results were obtained in several experiments using sulfanilamide, sulfapyridine or sulfathiazol together with azochloramid. It may be concluded, therefore, that the combined use of these sulfamido compounds and azochloramid exerts greater anti-pneumococcal activity than either drug alone in like or even higher concentrations.

Experiment V: The combined bacteriostatic activity of azochloramid and sulfathiazol, alone and in combination, upon staphylococcus aureus hemolyticus. As in the experiments on pneumococcus and Group A hemolytic streptococcus, the combined use of sulfamido compounds and azochloramid resulted in increased inhibition of growth of *staphylococcus aureus hemolyticus* beyond that exerted by either compound alone. A representative experiment ensues. Infusion broth containing sulfathiazol in concentrations from 0.1 to 100 mgm. per cent; broth containing azochloramid in concentration of 1:100,000; as well as broth containing both sulfathiazol (0.1 to 100 mgm. per cent) and azochloramid (1:200,000 and 1:300,000, respectively), were seeded with 0.2 cc. of a 1:10,000 diluted 18 hours broth culture of *staphylococcus aureus hemolyticus*. The tubes were incubated for 4 days at 37°C. and the growth was noted at various intervals. The results of this experiment are given in table 5.

Table 5 shows that the combined use of sulfathiazol and azochloramid delayed the growth of the organism more definitely than either drug alone. It is interesting to note that in combination with azochloramid sulfathiazol

TABLE 4

Bacteriostatic activity of azochloramid and sulfadiazine, alone and in combination, upon *pneumococcus type I* in infusion broth

HOURS OF INCUBATION AT 37°C.	SULFADIAZINE (MG. PER CENT)						SULFADIAZINE, MG. PER CENT AND AZOCILORAMID 1:200,000						SULFADIAZINE, MG. PER CENT AND AZOCILORAMID 1:400,000						AZOCILORAMID 1:150,000
	0		0.1	1	10	100	0		0.1	1	10	100	0		0.1	1	10	100	
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1) 18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2) 24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3) 42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4) 72	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5) 96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6) 144	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

—, no visible growth; + to + + + +, various degrees of visible growth.

TABLE 5

Bacteriostatic activity of azochloramid and sulfathiazol, alone and in combination, upon *Staphylococcus aureus hemolyticus* in infusion broth

HOURS OF INCUBA- TION AT 37°C.	SULFATHIAZOL (MG. PER CENT)						SULFATHIAZOL, MG. PER CENT AND AZOCHLORAMID 1:200,000						SULFATHIAZOL, MG. PER CENT AND AZOCHLORAMID 1:300,000						AZ CHL M 1:100
	0		0.1	1	10	100	0		0.1	1	10	100	0		0.1	1	10	100	
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1) 18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2) 28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3) 48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4) 96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

—, no visible growth; + to + + + +, various degrees of visible growth.

in lower concentrations (1 and 10 mgm. per cent) exerted greater bacteriostatic activity than in higher concentrations (100 mgm. per cent) either alone or in conjunction with azochloramid. Thus far this particular finding was obtained with the staphylococcus only. Essentially the same results were obtained in several experiments by the combined use of sulfathiazol or sulfadiazine and azochloramid.

DISCUSSION. Synergistic action of anti-microbial agents is a well known phenomenon. It suffices to mention the combined treatment of syphilis with arsenicals, bismuth and mercury. Such combinations of drugs may have definite advantages, provided that they are more effective than treatment with a single chemotherapeutic agent or cause fewer toxic reactions. It is interesting to note that *in vitro* experiments on the effectiveness of sulfamido compounds used in conjunction with other anti-microbial agents yielded different results: In some instances no evidence was obtained of any synergistic action; in others, as in the above reported experiments on sulfamido compounds and azochloramid, increased bacteriostatic effects resulted from the combined use of two drugs. Azochloramid in suitable concentration used together with certain sulfamido compounds exerted greater bacteriostatic activity toward pneumococcus type I, Group A hemolytic streptococcus, Group D hemolytic streptococcus, and *staphylococcus aureus hemolyticus* than did either compound alone in like concentration. Moreover, with certain microorganisms, these drugs, when used in combination, gave better results than several times the concentration of either agent alone. Thus these experiments present examples of the phenomena of synergism and potentiation. It is interesting to note that in order to obtain synergistic effects by the combined use of azochloramid and sulfamido compounds, a relatively large fraction of the effective concentration of the former drug had to be used, whereas a small fraction of the effective concentration of the sulfamido compounds frequently sufficed.

SUMMARY

An *in vitro* study on the combined antibacterial activity of sulfamido compounds and azochloramid revealed the following:

1. Azochloramid used in combination with sulfanilamide, sulfathiazol, and and sulfadiazine may exert greater bacteriostatic activity toward Group A hemolytic streptococcus than does either compound alone in like or somewhat higher concentrations.

2. A slight increase in bacteriostatic activity toward Group D hemolytic streptococcus was obtained when azochloramid was used in conjunction with sulfanilamide.

3. The combined use of azochloramid and sulfanilamide, sulfapyridine, sulfathiazol or sulfadiazine resulted in greater inhibition of growth of pneumococcus type I than did that of either drug alone in like or somewhat higher concentrations.

4. The combined use of sulfathiazol or sulfadiazine and azochloramid retarded the growth of *staphylococcus aureus hemolyticus* to a greater extent than did either drug in like or higher concentrations.

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EFFECT OF TREATMENT WITH TESTOSTERONE PROPIONATE ON MERCURIC CHLORIDE POISONING IN RATS¹

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Increase in kidney weight following injection of androgens has been reported repeatedly (1, 2, 3). It is more marked in mice than in rats, and in male than in female animals. Since the increase in dry weight of the kidney is of the same order as the increase in wet weight, there is a true renal hypertrophy and not simply edema. Testosterone propionate has also been shown to increase the degree of compensatory hypertrophy after unilateral nephrectomy (4), and to delay the atrophy which follows ligation of the ureter (5). In an attempt to ascertain whether the renal trophic effects were of any functional significance, Selye (6) gave female mice daily injections of testosterone (3-4 mgm.) for six days and then mercuric chloride (12.5-16.5 mgm. per kilogram) daily, or twice daily for five to eight days. In one experiment, the controls were all dead by the eighth day, while eight of ten treated animals recovered.

These results were sufficiently striking to suggest the possible value of testosterone in cases of mercuric chloride poisoning in man. Unfortunately, "pre-treatment" would rarely be possible. It seemed appropriate, therefore, to determine whether testosterone exerted a similar protective action when given at the same time as, or after, mercuric chloride.

METHODS. Male albino rats of a single strain weighing from 250-350 grams were used. In each group the control and treated animals were paired as closely as possible with respect to weight and age. Food and water were constantly available. A single injection of mercuric chloride, 6-12 mgm. per kilogram, in distilled water was given subcutaneously. The treated animals received testosterone propionate dissolved in sesame oil (Oreton)² in doses of 2.5-25 mgm. per rat immediately afterward, but the injection was, of course, made at a different site. The dose of testosterone propionate was repeated daily until the animal died or for seven days. Blocks of kidneys were fixed in formalin, and histological sections stained with hematoxylin and eosin.

In order to determine the effect of testosterone propionate alone, 5, 10, and 25 mgm. were injected daily for one week; two rats were used for each dose. The animals showed no abnormal symptoms. One of each group was killed 24 hours after the last dose. The kidneys weighed 735, 925, and 1200 mgm. per 100 grams of rat, in the order of increasing dose.

¹ Supported by a grant from the Commonwealth Fund.

² Dr. Max Gilbert of the Schering Corporation generously furnished the Testosterone Propionate (Oreton) in these experiments.

age. Histologically, the tubule cells showed slight cloudy swelling without evidence of abnormal hyperplasia. The other rats were sacrificed eight days after the last dose of testosterone. Kidney weight was 1070, 850, and 1070 mgm. per 100 grams of rat. Sections were microscopically normal.

RESULTS. The results are summarized in table 1. After a dose of 12 mgm. of mercuric chloride per kilogram no animals survived. While the average duration of life was slightly greater in the group receiving 10 mgm. testosterone propionate daily, the difference is not statistically significant; nor is the huge dose of 25 mgm. per rat per day any more effective than 5 mgm.

Following a dose of 8 mgm. of bichloride per kilogram a few treated animals survived while all control animals died. The survival with doses of 2.5 and 5 mgm. of testosterone is not significant with such small series. The survival

TABLE 1
Effect of testosterone propionate on mercuric chloride poisoning

HgCl ₂	TESTOSTERONE PROPIONATE	NUMBER OF RATS	NUMBER SURVIVED	MEAN DURATION OF LIFE OF NON-SUR- VIVERS
mgm./kgm.	mgm./rat/day			hours
12	0	18	0	84
12	5	6	0	87
12	10	6	0	113
12	25	6	0	98
8	0	17	0	99
8	2.5	12	2	111
8	5	14	1	138
8	10	13	4	151
6	0	12	2	121
6	5	12	9	128

of four of thirteen animals receiving 10 mg. testosterone propionate daily is, however, apparently significant, for the difference in survival is 2.7 times its standard error. With this dose of mercuric chloride, larger doses of testosterone seem more beneficial than smaller ones in contrast to the results with 12 mgm. per kilogram doses of mercuric chloride. The mean duration of life in the animals which received 5 and 10 mg. testosterone was significantly longer than in the control group.

When the dose of mercury was reduced to 6 mgm. per kilogram two of twelve control animals survived while nine of twelve treated with 5 mgm. testosterone propionate daily survived. This difference is 2.9 times its standard error and is therefore significant.

Since the animals drank relatively little water after poisoning (the treated rats no more than the controls), and since the maintenance of a large urine volume is apparently of benefit during the period of tubular regeneration (7), a

group of twelve rats was given 8 mgm. of mercuric chloride per kilo, 10 mgm. testosterone propionate on alternate days, and 30-40 cc. of physiological salt solution intraperitoneally daily after the third day. Urine volumes were 5-50 cc. but only two of the animals survived.

No survivals occurred in a group of six rats poisoned with 8 mgm. of mercuric chloride per kilogram and given a single injection of 10 mgm. testosterone propionate.

All of the animals lost weight after the injection of mercuric chloride. The amount lost varied considerably in different rats, those surviving for the longer intervals usually showing the greatest loss. The rate of loss averaged 1 to 2 per cent of the original weight per day in control groups, and from 1.2 to 3.4 per cent of the original weight in the treated groups. The kidneys were enlarged in both control and treated groups. In a sample of twelve normal male rats of the same strain, age, and weight as the animals used, the mean kidney weight per 100 grams body weight was 841 mg. In the animals dying after receiving mercuric chloride only, the mean kidney weight per 100 grams of body weight was 1,116 mg., while in the treated animals it was 1,236 mgm. This difference is not statistically significant, nor was there any significant difference in the kidney weights of animals receiving 6, 8, or 12 mgm. of mercuric chloride per kilogram, or the various amounts of testosterone. It is impossible to say whether the increased weight is more than edema since estimations of dry weight were not made. The kidney weight in the treated animals exceeds that in the normal group by approximately the same degree as Ludden and Kruegar (2) found from daily injections of testosterone. The average kidney weight in animals surviving poisoning, and killed ten weeks later, was 871 mgm. per 100 grams of rat.

Microscopically,³ there were similar changes but of different degrees in the kidneys of rats dying after treatment with testosterone and in the controls. The difference between the two groups was not striking, but as a result of careful study of the slides from both groups, mixed together, it was possible objectively to pick out most, though not all, of the kidneys of testosterone-treated rats. The latter showed, in general, less extensive necrosis of tubules, a greater amount of well-preserved tubular epithelium, and less calcification than those of the non-treated animals. A slight difference in regenerative capacity of the cells lining the tubules was also noticed between the two groups. In the group treated with testosterone, there were more bizarre and large nuclei as well as more mitoses in the cells lining the convoluted tubules. These changes were interpreted as indicating an attempt at regeneration and were more abundant in the kidneys of testosterone-treated rats.

Some of the kidneys of surviving animals killed ten weeks after poisoning

³ Dr. Harry Goldblatt and Dr. S. Koletsky of the Institute of Pathology, Western Reserve University kindly examined these sections.

presented an essentially normal picture while others showed a focal interstitial nephritis characterized by occasional dilated tubules containing hyaline material. Some kidneys showed the flattened tubular epithelium described by MacNider (8) in animals recovered from uranium poisoning and which he has shown to be resistant to subsequent injury by mercury. In some kidneys small deposits of calcium were present in the lining epithelium of the tubules and in the tubular lumen.

Treatment of mercuric chloride poisoning in rats with testosterone propionate is ineffectual when large doses of mercury are given. When, however, the dose of mercury is near the minimal fatal dose, such treatment leads to the survival of an increased proportion of animals. Since the mortality of mercuric chloride poisoning in man has not been significantly improved by the various antidotes proposed, it seems that a trial of testosterone is justified on the basis of the experimental work available. The outcome of a single case, however, depends on so many factors, and is so unpredictable when the patient is first seen, that the value of any new treatment can only be assayed on the basis of a series of cases.

CONCLUSIONS

Male albino rats poisoned with 6, 8, and 12 mgm. of mercuric chloride per kilogram were treated with 2.5 to 25 mgm. of testosterone propionate per rat per day. With the largest dose of mercury no animals survived. Thirty per cent of animals poisoned with 8 mgm. mercuric chloride per kilogram and receiving 10 mg. per day of testosterone survived, while with 6 mgm. mercuric chloride per kilogram 75 per cent of the treated animals survived compared to 17 per cent of controls.

Treatment with testosterone propionate apparently increases the survival rate of rats poisoned with small doses of mercuric chloride, but is without effect when larger doses of poison are used.

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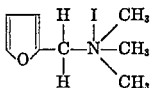
THE COMPARATIVE PHYSIOLOGICAL ACTION OF ANALOGOUS TRIMETHYL AMMONIUM IODIDES OF BENZENE AND FURANE^{1, 2}

EDWIN J. FELLOWS AND A. E. LIVINGSTON

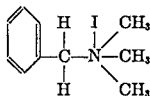
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It long has been known that a similarity in physical and chemical properties exists between compounds of benzene and analogous heterocyclic derivatives. In 1925 Gilman and Pickens (1) concluded, as a result of local anesthetic studies on derivatives of benzene and corresponding furane, thiophene and pyrrol compounds, that a similarity in physiological action also existed between these groups of compounds. Gilman and his associates found this parallelism applicable also to certain modifications of benzene and analogous heterocyclic agents with sweetening (2) and hypnotic (3) properties. Phatak and Leake (4, 5) found these correlations could be extended to include certain bacteriostatic benzene and furane analogues. Recently Alles and Feigen (6) reported certain similarities in the type of physiological effects produced by isopropylamines of thiophene, furane and benzene. Extension of these correlation studies to compounds with different types of action would appear desirable. The present studies therefore were carried out to determine if generalizations could be made concerning similarities and differences in the type of physiological action produced by certain analogous ammonium iodides of benzene and furane.



Furfuryl Trimethyl Ammonium
Iodide (F T M)



Benzyl Trimethyl Ammonium
Iodide (B T M)

Furfuryl trimethyl ammonium iodide (F T M) previously was found to produce a parasympathetic type of effect (7) and to manifest a relatively

¹ This investigation was supported in part by the D. J. McCarthy Foundation.

² We wish to express our thanks and appreciation to Drs. F. P. Nabenhauer and Glenn L. Ulyot of Smith, Kline and French Laboratories for samples of the compounds used in the present studies.

mild "nicotine-stimulating" action after atropine (8). Luchsinger (9) stated that benzyl trimethyl ammonium (B T M) caused cardiac and glandular effects similar to muscarine and that its cardiac action could be antagonized by atropine. Lee, Van Arendonk and Chen (10) found benzyl trimethyl ammonium iodide exhibited marked depressor activity which was antagonized by atropine. These authors also state that the benzyl compound possessed a relatively mild "nicotine-stimulating" action.

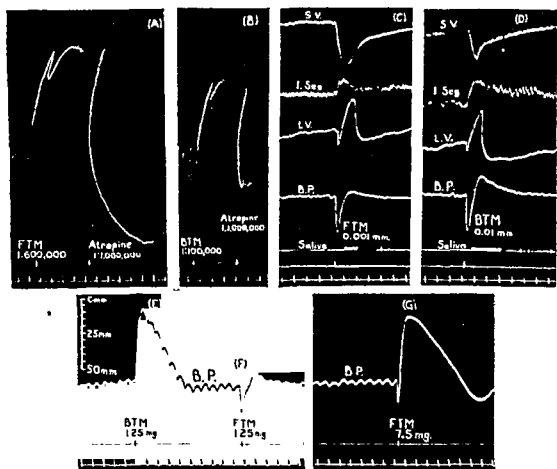
ISOLATED PERFUSED GUINEA-PIG LUNGS. Bronchial constriction after furfuryl- and benzyl-trimethyl ammonium iodides appeared probable because of the nature of their previously reported actions. Comparisons therefore were made of the effect of these compounds on perfusion fluid flow through isolated guinea-pig lungs. The method used was similar to that described by Sollmann and Von Oettingen (11). Addition of the furfuryl compound in 0.01 mgm. quantities caused a 17 per cent, and in 0.1 mgm. amounts a 30 per cent decrease (average figures) in rate of perfusion fluid flow through the lungs. Comparable decreases in rate of perfusion fluid flow through the lungs were obtained with the benzyl compound in a dose range of 0.1 to 1.0 mgm. Both compounds were ineffective after atropine.

ISOLATED INTESTINE. A definite increase in tonus was reported previously (7) with 1:5,000,000 furfuryl trimethyl ammonium iodide in Tyrode's solution surrounding a segment of isolated rabbit intestine. Comparable effects have been obtained in the present studies with 1:750,000 to 1:1,000,000 benzyl trimethyl ammonium iodide. Larger amounts of these compounds induced marked spasm which was antagonized by atropine. This is illustrated in figure 1 in which spasm after 1:600,000 F T M (fig. 1, A) and 1:100,000 B T M (fig. 1, B) is shown to be antagonized by 1:1,000,000 atropine. In other experiments antagonism to augmentation after 1:1,000,000 furfuryl- or 1:100,000 benzyl-trimethyl ammonium iodide was obtained with concentrations of atropine sulfate as low as 1:100,000,000.

CIRCULATION. *Before atropine.* The marked similarity in type of effect produced by furfuryl- and benzyl-trimethyl ammonium iodides in etherized dogs is illustrated in figure 1 (C and D). Intravenous injection of 0.001 millimol of F T M per kgm. (fig. 1, C) caused a decrease in spleen volume, increased tonus of a segment of intestine, an increase in limb volume, fall in blood pressure and a marked flow of saliva. In the same animal 0.01 millimol of B T M per kgm. (fig. 1, D) induced an identical type of response. In numerous experiments on etherized cats the depressor activity of the furfuryl compound was found to be 6 to 8 times greater than that of the benzyl derivative. The depressor action of both compounds was antagonized by atropine sulfate.

After atropine. As mentioned above it has been reported that benzyl- (10) and furfuryl- (8) trimethyl ammonium iodides exhibited relatively mild "nicotine-stimulating" properties. In the present experiments comparisons

of the circulatory activity of these compounds were made in decapitated cats which received an initial injection of 3.0 mgm. of atropine sulfate per kgm. and supplementary doses of 1.5 mgm. per kgm. 15 minutes prior to administration of either the benzyl or furfuryl derivative. In figure 1 it is shown that

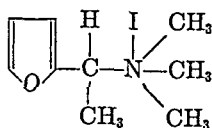


E, F and G Cat 3.7 kgm. anesthetized with nembutal, decapitated and atropinized. B. P. = carotid blood pressure by mercury manometer. Upper line indicates injection into a femoral vein, lower line = time in minutes. Doses are in millimol per kgm.

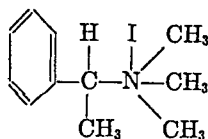
E, F and G Cat 3.7 kgm. anesthetized with nembutal, decapitated and atropinized. B. P. = carotid blood pressure by mercury manometer. Upper line indicates injection into a femoral vein. Lower line = time in minutes. Doses are in mg. per kgm.

1.25 mgm. of B T M per kgm. (fig. 1, *E*) caused a marked rise of blood pressure in a decapitated, atropinized cat, whereas 1.25 mgm. of F T M per kgm. (fig. 1, *F*) produced only a fall of blood pressure followed by a slight rise. In this animal 7.5 mgm. of F T M per kgm. (fig. 1, *G*) were required to cause a rise of blood pressure comparable with that observed after 1.25 mgm. of B T M per kgm. In similar experiments on 7 other cats the benzyl derivative

was found to exhibit 6 to 8 times as great a pressor activity, after atropine, as its furfuryl analogue. The quantitative relationship which exists between these compounds for parasympathetic type of effect, therefore, is reversed in the case of their pressor action, after atropine.



α -furfuryl ethyl trimethyl ammonium iodide (α -F)



α -phenyl ethyl trimethyl ammonium iodide (α -B)

Analogous modifications of F T M and B T M, namely α -furfuryl ethyl trimethyl ammonium iodide (α -F) and α -phenyl ethyl trimethyl ammonium

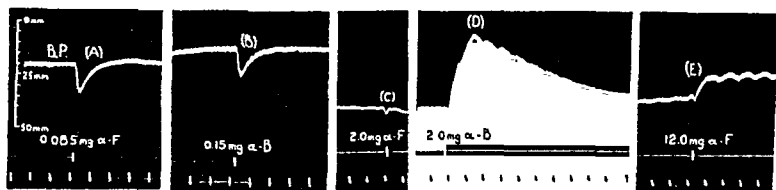


FIG. 2. A and B. Cat 3.3 kg., ether anesthesia. B. P. = carotid blood pressure by mercury manometer. Upper line indicates injection into a femoral vein. Lower line = time in minutes. Doses in mg. per kgm.

C, D and E. Cat 5.0 kg., anesthetized with nembutal, decapitated and atropinized. B. P. = carotid blood pressure by mercury manometer. Upper line indicates injection into a femoral vein. Lower line = time in minutes. Doses in mgm. per kgm.

iodide (α -B) also have been included in the present studies. The α -phenyl compound (bromide) was reported by Hunt and Renshaw (12) to exhibit less "muscarinic" and "nicotine-stimulating" activity than β -phenyl ethyl trimethyl ammonium bromide. The depressor effectiveness of the α -phenyl derivative, therefore, should be less than that of B T M because Lee et al. (10) report the β -phenyl compound possessed a lower order of depressor activity than B T M. The α -furfuryl derivative is a new compound and has not been investigated previously. Quantitative comparison of effects after the α -furfuryl and α -phenyl derivatives, other than those on circulation, appeared impractical. For example, increased tonus of isolated rabbit intestine was observed with these compounds but occasionally both produced depression. In isolated perfused guinea-pig lung experiments the α -furfuryl derivative caused a decrease in rate of perfusion fluid flow but the α -phenyl compound was ineffective in the amounts used (up to 100 mgm.). In small doses the α -furfuryl compound usually evidenced slightly greater depressor activity than its phenyl analogue as illustrated in figure 2 where it is shown

0.085 mgm. α -F per kgm. (fig. 2, A) produced a fall of blood pressure in an etherized cat which is comparable with that caused by 0.15 mgm. of α -B per kgm. (fig. 2, B). We have observed, as did Hunt and Renshaw (12), that while depressor effects are demonstrable with small doses of α -phenyl, large doses of this compound may produce pressor effects. After atropine, the intensity of α -phenyl pressor action was found to be much greater than that of its furfuryl analogue as illustrated in figure 2 where it is shown that 2.0 mgm. of α -F per kgm. (fig. 2, C) in a decapitated, atropinized cat caused little if any change in blood pressure whereas in the same animal 2.0 mgm. of α -B per kgm. (fig. 2, D) produced a marked rise of blood pressure. A slight pressor response was obtained in this animal with a dose of 12.0 mgm. of α -F per kgm. (fig. 2, E). The intensity of depressor action, as well as pressor action after atropine, of the α -furfuryl and α -phenyl compounds was less than that of their parent homologues.

DISCUSSION. While similarities in physiological action have been reported for certain benzene and furane analogues, indications of notable differences also have been obtained. For example, in anesthetized dogs Alles and Feigen (6) found furyl- and phenylisopropylamine both produced a rise of blood pressure but observed that the furyl compound exhibited less activity and also manifested an initial depressor effect which was diminished by atropine. When studied with respect to its action upon isolated intestine these authors state the tendency of the furyl derivative toward a parasympathetic type of effect, relative to that of its phenyl analogue, was very noticeable. In the present studies furfuryl- and benzyl-trimethyl ammonium iodides were found to produce certain physiological effects which qualitatively were indistinguishable but quantitatively several outstanding differences were evident. Both compounds manifest a parasympathetic type of effect as shown by the fact that their bronchial, intestinal, salivary and depressor action was prevented or overcome by atropine, but a predilection toward this type of action appears to exist in the case of the furfuryl derivative since it exhibited 6 to 8 times the activity of its benzene analogue. The qualitative similarity in action between the furfuryl and benzyl compounds also is indicated by the fact that both produced a rise of blood pressure in atropinized cats but the reversal in quantitative relationship between the two compounds for this type of action suggests a more pronounced tendency on the part of the benzyl derivative to evoke a pressor type of response.

Insofar as circulatory effects in anesthetized animals are concerned α -furfuryl ethyl- and α -phenyl ethyl- trimethyl ammonium iodides exhibited similarities and differences approximating those of their parent homologues. The α -furfuryl and α -phenyl compounds both caused depressor effects in small doses before atropine, and pressor effects in large doses after atropine. The α -furfuryl derivative, however, manifested slightly greater depressor activity

than its benzene analogue whereas the latter evidenced a much more pronounced tendency to induce pressor effects.

SUMMARY

1. In the present studies furfuryl- and benzyl-trimethyl ammonium iodides exhibited qualitatively indistinguishable but quantitatively different physiological effects. A parasympathetic type of effect was demonstrated for both compounds but the furfuryl derivative was found to be 6 to 8 times more active than its benzyl analogue. Both derivatives produced a rise of blood pressure in atropinized cats but in this case the benzyl compound was 6 to 8 times more active than the furfuryl derivative.

2. In anesthetized dogs and cats α -furfuryl ethyl trimethyl ammonium iodide evidenced a slightly greater depressor potency than α -phenyl ethyl trimethyl ammonium iodide whereas the latter exhibited greater pressor activity after atropine than its furfuryl analogue.

3. The intensity of depressor action, as well as pressor action, after atropine, of α -furfuryl ethyl- and α -phenylethyl trimethyl ammonium iodides was found to be less than that of their parent homologues.

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CONJUGATION OF SULFANILAMIDE BY A PATHOLOGICAL TISSUE IN VITRO*

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It is generally accepted that the conjugation of sulfanilamide takes place in the liver. Stewart *et al.* (1) found that the removal of the liver and gastrointestinal tract in the rabbit prevented the conjugation of sulfanilamide. Van Winkle and Cutting (2) confirmed these findings in the rabbit and they showed that in the cat the removal of the liver and spleen prevented the formation of conjugated sulfanilamide. Harris and Klein (3, 4) have shown that sliced rabbit liver was able to transform sulfanilamide into the acetyl derivative, while muscle, spleen, kidney, and blood failed to do so.

During the course of chemotherapeutic experiments our attention was called to the fact that the distribution of conjugated sulfanilamide was not uniform in all the tissues of chicks infected with fowl-pox virus. While the ratio between free and total sulfanilamide was approximately the same in the muscle, normal skin and blood, the amount of conjugated sulfanilamide was significantly higher in the fowl-pox lesion. It was the object of the present study to ascertain whether the epithelioma induced by fowl-pox virus in the chicken has the capacity to conjugate sulfanilamide in vitro.

MATERIAL AND METHODS. Chicks, 2 to 4 weeks old, were inoculated with an emulsion of chorio-allantoic membranes, containing fowl-pox virus. The site of the inoculation was the dorsal aspect of the head. The feathers were removed previous to the inoculation. From 7 to 16 days following the inoculation, after confluent lesions appeared on the skin, the chicks were killed and the fowl-pox lesion and the liver were removed immediately. In some experiments pieces of normal skin were removed also. The tissues thus removed were sliced with a hand razor. The thickness of the sections was approximately 0.5 mm. The slices were placed in Erlenmeyer flasks, which contained 10 or preferably 5 cc. of Ringer-phosphate (5), Ringer-bicarbonate (6) or Ringer solution with sodium acetate buffer. Dextrose was added in the concentration of 200 mgm. per 100 cc. and sulfanilamide in the concentration of 20 mgm. per 100 cc.

The flasks were placed in a constant temperature water bath at 39°C., and were shaken by a Warburg shaking apparatus for 5 hours. After this period the fluid of each flask was analyzed for free and total sulfanilamide according to the Bratton-Marshall method (7). A photoelectric colorimeter was employed in these determinations. The conjugating capacity of the slices was recorded in milligrams of sulfanilamide converted

* This work was aided by a grant from the Mallinckrodt Chemical Works.

into the conjugated drug by 1 mgm. of tissue (dry weight) in 5 hours. The dry weight of the tissue slices was in the range of 40 to 230 mgm.

The flasks were aerated in the experiments in which Ringer-phosphate or Ringer-acetate were employed. A gas mixture of 95 per cent oxygen and 5 per cent CO_2 was used in the series in which Ringer-bicarbonate was employed.

RESULTS. The results of our *in vitro* experiments are presented in the table. It can be easily seen that there is a significant difference between the conjugating capacity of the fowl-pox lesion and that of the normal skin. In the 4 cases in which the normal skin seemed to conjugate sulfanilamide, the difference between free and total sulfanilamide was less than 5 per cent of the total sulfanilamide, a difference which can hardly be considered significant. The conjugating capacity of the slices varied greatly. The mean values of conjugated sulfanilamide for fowl-pox slices and for liver slices did not differ significantly.

Crabtree (6) estimates that the growth tissue in the fowl-pox lesion represents only 50 per cent of the total weight, the rest being made up by fat and other normal components of the skin. If we assume that the growth tissue in the fowl-pox lesion represents a smaller percentage of the total weight than the percentage of active tissue in the liver, then the conjugating capacity of the fowl-pox lesion should be considerably greater than that of the liver per unit of active tissue.

The amounts of conjugated sulfanilamide produced by the chicken liver are essentially the same as those found by Klein and Harris (4) in rabbit liver. The variability of the conjugating capacity in animals of the same species has also been pointed out by the same investigators.

DISCUSSION. Our experiments indicate that the fowl-pox lesion has the ability to conjugate sulfanilamide *in vitro*. The exact nature of this conjugating capacity is not clear. Our attempts to extract an enzyme gave negative results, and tissue pulp made out of fowl-pox lesions failed to conjugate sulfanilamide when submerged in a solution of the drug and maintained at 39°C. for 24 hours.

Klein and Harris (4) have shown that the *in vitro* acetylation of sulfanilamide is limited by the ability of the tissue to form acetate. Conjugation could be increased by the addition of acetate or other substances, which may be transformed into acetate, for example, pyruvate or aceto-acetate. On the basis of these facts it is reasonable to assume that the ability to conjugate sulfanilamide, possessed by the fowl-pox lesion, is a result—at least in part—of the ability of the tissue to produce acetate. Since normal chicken skin does not conjugate sulfanilamide significantly, the appearance of the conjugating capacity in the lesion may represent an expression of the altered metabolism associated with the active proliferation of the epithelium.

Crabtree (6) reported that the fowl-pox lesion belongs to that group of tissues which possesses both anaerobic and aerobic glucose-splitting power.

TABLE 1

CHICK NUMBER	METABOLIC SOLUTION	MG. OF SULFANILAMIDE CONJUGATED PER 1 MG. DRY TISSUE		
		Fowl-pox lesion	Liver	Normal skin
1	Ringer-acetate	0.0035	0.0030	0
2		0.0034	0	0
3		0.0004	0.0030	0
4		0	0.0017	0
5		0.0040	0.0015	0.0003
6		0.0026	0.0012	0
7		0.0020	0	0
8		0.0025	0.0015	0
9		0.0037	0.0017	0
10		0.0014	0.0021	0.0003
11		0.0016	0	0.0007
12		0.0006	0.0006	0
13		0.0016	0.0020	0
14		0.0019	0.0002	0.0013
15		0	0.0028	
16		0.0008	0.0002	
17		0.0006	0.0018	
18		0.0012	0.0002	
	<i>Mean value</i>	<i>0.0018</i>	<i>0.0013</i>	<i>0.00018</i>
19	Ringer-phosphate	0.0010	0.0017	
20		0.0004	0.0033	
21		0.0023	0.0025	
22		0.0050	0.0005	
23		0.0009	0.0008	
24		0.0010	0.0014	
25		0.0024	0.0015	
26		0.0028	0.0016	
27		0	0.0022	
28		0.0015	0.0008	
	<i>Mean value</i>	<i>0.0017</i>	<i>0.0016</i>	
29	Ringer-bicarbonate	0.0022	0.0021	
30		0.0014	0.0011	
31		0.0007	0.0012	
32		0.0009	0.0015	
33		0.0010	0.0018	
34		0	0.0014	
35		0	0.0016	
36		0.0010	0.0019	
37		0.0008	0.0014	
38		0.0020	0.0007	
39		0.0013	0.0013	
40		0.0017	0.0019	
	<i>Mean value</i>	<i>0.0011</i>	<i>0.0015</i>	

Since this glucose splitting means formation of lactic acid, a probable precursor of pyruvic and acetic acid, it is not unlikely that there is a certain relationship between the carbohydrate metabolism of the fowl-pox lesion, as reported by Crabtree, and the conjugating capacity of this tissue. The conjugating capacity may be worth considering in connection with other pathological growths and malignant tumors.

SUMMARY

Fowl-pox lesion is able to conjugate sulfanilamide *in vitro*. The relationship between this conjugating capacity and the glucose-splitting power of the fowl-pox lesion is discussed.

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SOME TOXICOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF GRAMICIDIN, TYROCIDINE AND TYROTHRIN

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Increased attention has recently been directed towards a group of bacterial chemotherapeutic agents derived from soil microorganisms. These substances have been shown to possess marked bacteriostatic and bactericidal properties against gram-positive and in some instances against gram-negative microorganisms (1, 2, 3, 4). The available reports, dealing primarily with the isolation, chemical properties and antibacterial efficacy of these agents, show that the activity of the crude preparation (tyrothricin) is due to two substances designated as gramicidin and tyrocidine. Further investigation indicated that tyrothricin contains approximately 85 per cent tyrocidine and 15 per cent gramicidin, the latter showing the greatest activity *in vivo*. Little however has been published regarding the pharmacological or toxicological properties of these substances, knowledge of which becomes increasingly desirable in view of the steadily mounting number of clinical investigations.

The present communication is concerned with an investigation of the acute and cumulative toxicity and certain pharmacological properties of gramicidin, tyrocidine and tyrothricin. Since most of the clinical studies are being conducted with tyrothricin, our studies were mainly concerned with this agent.

TECHNIQUE. The materials used in our experiments consisted of pure gramicidin, tyrocidine and a crude preparation of tyrothricin.¹ Since these substances have been shown to be stable in alcoholic solution as well as in the dry state, stock solutions were prepared in ethyl alcohol. For the injection into animals suspensions were made by adding the alcoholic stock solution to sterile saline or 1 per cent gum acacia. Since the latter vehicle permitted suspensions of greater homogeneity and stability it was used in the greater part of the experiments. Control toxicity studies with gum acacia showed it to be non-toxic when given intraperitoneally in doses as large as 25 grams per kilogram. Homogeneity was obtained by means of a high speed electrical mixer as well as by thorough shaking by hand. The reaction of all suspensions was neutral (pH 7.4-7.6) as determined by a glass electrode.

EXPERIMENTAL. *Acute toxicity.* The acute toxicity of gramicidin, tyrocidine and tyrothricin was determined in a series of 1200 swiss mice and 210 albino rats

¹ The materials used in these experiments were prepared by Dr. Max Tishler from cultures supplied by Dr. J. L. Stokes of the Research Laboratories of Merck and Company, Inc.

by peroral, intravenous and intraperitoneal administration. Doses ranging from 1 to 30 mgm. per kilogram were given intravenously and from 2.5 to 90 mgm. per kilogram intraperitoneally. Observations were made frequently during the first 10 hours and thereafter once daily for 7 additional days. Tissues of animals dying during this period were retained for histological study.

TABLE 1
Intravenous toxicity of gramicidin, tyrocidine and tyrothricin for mice

	MG./KGM.	NUMBER OF MICE	PER CENT MORTALITY* TIME IN DAYS		
			1	2	7
Gramicidin.....	0.625	10	0	0	0
	1.25	10	0	0	0
	2.50	10	33	36	40
	3.75	10	86	93	100
	5.00	10	100	100	100
	10.00	10	100	100	100
	15.00	10	100	100	100
Tyrocidine.....	1.25	10	0	0	2
	2.50	10	0	0	10
	5.00	10	0	0	18
	10.00	10	0	0	26
	15.00	10	15	15	45
	17.50	10	77	77	85
	20.00	10	95	95	96
	25.00	10	100	100	100
	30.00	10	100	100	100
Tyrothricin.....	1.20	10	0	0	9
	2.50	10	10	11	28
	3.75	10	29	38	60
	5.00	10	86	93	100
	10.00	10	100	100	100
	20.00	10	100	100	100

* Determined according to the method of Burns (6).

In the interest of space only the results obtained in mice are presented in tables 1 and 2 since the findings in rats were essentially the same.

None of the preparations was toxic when administered by mouth to mice or rats in doses as large as 1000 mgm. per kilogram. The lack of oral toxicity might be due to the insolubility of these agents in water. However, upon intraperitoneal and intravenous injection all preparations proved to be definitely toxic, gramicidin and tyrothricin considerably more so than tyrocidine. Following intravenous administration of gramicidin, death occurred most frequently within the first 24 hours, whereas with tyrothricin and especially tyro-

cidine a large percentage of deaths occurred 3 to 4 days after the injection. Whether the much more rapid onset of toxic manifestations and death after intravenous injection of gramicidin suspensions is at least in part due to physical factors, such as large particle size, cannot as yet be decided and is under further investigation.

TABLE 2

Intraperitoneal toxicity of gramicidin, tyrocidine and tyrothricin for mice

	MG/KG.	NUMBER OF MICE	PER CENT MORTALITY TIME IN DAYS		
			1	2	7
Gramicidin.....	5.0	10	0	0	0
	10.0	10	0	0	0
	20.0	10	0	2	4
	30.0	10	0	3	11
	40.0	10	0	10	53
	50.0	10	0	24	83
	60.0	10	17	43	100
	75.0	10	38	74	100
Tyrocidine.....	2.5	10	0	0	0
	5.0	10	0	0	0
	10.0	10	0	0	0
	20.0	10	0	0	0
	35.0	10	0	0	5
	40.0	10	10	14	44
	60.0	10	35	39	72
	70.0	10	71	72	96
Tyrothricin.....	90.0	10	95	96	100
	10.0	50	0	0	16
	20.0	50	0	0	63
	30.0	50	0	10	89
	40.0	50	20	20	94
	50.0	50	50	56	97
	60.0	50	60	60	99
	70.0	50	60	80	99
	80.0	50	90	100	100
	90.0	50	100	100	100

Upon intraperitoneal administration tyrothricin appeared to be the most toxic of the three preparations. With all preparations both the intravenous and intraperitoneal toxicity appeared considerably greater when observations were extended over a 7-day period (tables 1 and 2).

Toxic signs, consisting of restlessness followed by depression, appeared 1-2 hours after intraperitoneal injection of lethal doses. In large doses all three

preparations cause a fall in body temperature, while therapeutic doses have no such effect. In rats nonlethal doses frequently produce an anorexia with gradual loss of weight and muscular tone. In both species death appeared to be due to respiratory failure since the heart continued to beat for a considerable time after respiration had ceased.

Cumulative toxicity. The effect of repeated daily administration of gramicidin and tyrothricin was determined in a series of 10 dogs. For these studies the preparations were suspended in 5 per cent glucose and administered intravenously in doses of 2 and 4 mgm. per kilogram. Body temperature, heart and respiratory rate, and blood picture (red, white, differential count, hemoglobin and hematocrit) were observed at frequent intervals. The blood samples for analysis were taken prior to the injection of the suspensions.

Daily doses of 2 mgm. of gramicidin or tyrothricin per kilogram caused death in most dogs within 2-8 days. Gramicidin appeared somewhat more toxic than tyrothricin since most deaths following gramicidin administration took place after 2-3 injections whereas 5-6 injections of tyrothricin were tolerated. All dogs developed anorexia and lost weight; the latter effect became more pronounced as the experiment progressed. During and shortly following each injection most dogs secreted excessive amounts of saliva and showed a slight rise in body temperature. Shortly before death the body temperature decreased. No significant change in heart or respiratory rate took place until shortly before death, at which time the heart rate became slow and the respiration shallow.

Daily blood examinations showed that all dogs receiving 2-4 mgm. of tyrothricin per kilogram developed marked leucocytosis. Dogs which tolerated more than 10 consecutive doses of the drug became anemic, the erythrocyte count ranging from 2.06×10^5 to 3.95×10^5 cells per c.mm. One dog with marked leucocytosis and anemia returned to normal after a period of 2 months during which time no drug had been given. This might indicate that the anemia caused by daily injections of tyrothricin is related to the hemolytic properties, which it can display *in vitro* (5).

Gramicidin had no apparent effect upon the blood picture during the short period which the animal survived. However, equivalent doses of gramicidin might have a similar effect as tyrothricin if the animal could tolerate a larger number of consecutive doses.

*Pathology.*² "Upon autopsy there were generalized congestion with degenerative changes in all organs, fatty liver, and an occasional enlargement of the spleen. Ascites was found in most cases. Upon microscopic examination the following changes were found: The congestion in the spleen was most pronounced. If the spleen did not have an open circulation this might have readily been interpreted as hemorrhage into the organ. At times the congestion

² We are indebted to Dr. William Antopol of the Newark Beth Israel Hospital for the pathological findings.

appeared to be most pronounced in the perifollicular zone, but this was not a constant finding. The cells in the pulp were very few in those areas in which the cytoplasmic reticulum was present—it appeared to be undergoing severe degenerative changes and necrosis with karyorrhexis."

Effects on isolated organs. The effect of gramicidin, tyrocidine and tyrothricin was investigated in the isolated rabbit's intestine and the isolated frog heart. The pH of all solutions was between 7.4–7.6. Locke's solution was used for the experiments on isolated intestine and Ringer's solution for the Straub heart.

In the isolated rabbit's intestine tyrocidine in doses of 6.4 mgm. per 100 cc. of Locke's solution produced a marked contraction whereas similar doses of

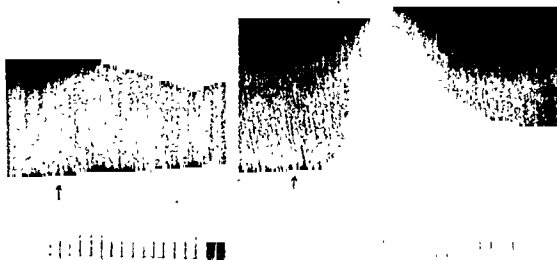


FIG. 1. ACTION OF GRAMICIDIN AND TYROCIDINE ON ISOLATED RABBIT INTESTINE

2. 32 micrograms Tyrocidine

gramicidin had no effect. This action was reversible since upon removal of the tyrocidine the intestine returned to normal within 15 minutes (fig. 1).

When tested in the isolated (Straub) frog heart, tyrocidine proved again to be the most active of the 3 preparations. The addition of 32 micrograms of tyrocidine suspended in frog ringer decreased both the rate and amplitude of the ventricular beat, and the heart finally stopped midway between systole and diastole (fig. 2). A similar effect was produced with a dose of 264 micrograms of gramicidin.

Blood pressure and respiration. The action of gramicidin, tyrocidine and tyrothricin upon the blood pressure and respiration was determined in cats and rabbits anesthetized with urethane; the usual methods of manometric recording and cannulation of the carotid artery were employed. Injections of the above

agents suspended in saline were made into the jugular vein and the blood pressure, respiratory rate and respiratory volume were recorded. All injections were made slowly, averaging approximately one cc. per minute. In most animals gramicidin, tyrocidine and tyrothricin produced a marked fall in blood pressure after the second or third consecutive injection of 1 mgm. per kilogram or more (fig. 3), while single injections, even of large amounts, frequently had no such effect. Some cats tolerated large doses before a drop in blood pressure was observed. In small doses tyrocidine depressed the respiration somewhat whereas comparable doses of gramicidin did not. With lethal doses of tyrocidine the respiration became irregular and stopped shortly before, or simultaneously with, the terminal fall of blood pressure and standstill of the heart.

Local effects on skin and mucous membranes. The clinical application of gramicidin and tyrothricin to mucous membranes led us to study their possible irritating properties on such tissues. This was done by instilling saline suspensions of both preparations into the conjunctival sacs of albino rabbits or by

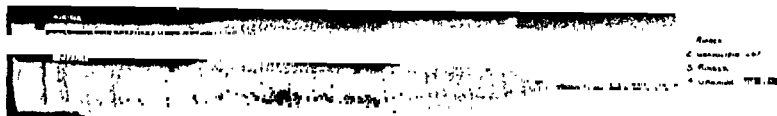


FIG. 2. EFFECT OF GRAMICIDIN AND TYROCIDINE (GRAMINIC ACID) ON THE ISOLATED FROG HEART

injecting 0.1 cc. subcutaneously or intradermally into the shaved abdomen of guinea pigs. No evidence of irritation was observed if concentrations of 500 micrograms per cc. of saline were applied to the cornea for 120 minutes. However, when the dry material was dusted into the eye sac there was marked irritation, characterized by edema and inflammation of the conjunctiva followed by a cloudiness of the cornea. These signs were still present 24 hours after installation. Application of an inert powder (barium sulfate) in a similar manner did not produce such an effect. The subcutaneous or intradermal injections produced local nodules which remained unabsorbed over a period of 5-6 weeks.

DISCUSSION. The foregoing data indicate that gramicidin and tyrothricin are more toxic than tyrocidine when injected intravenously and intraperitoneally in mice and rats, whereas none of the preparations is toxic upon single or repeated administration by mouth. The impossibility of preparing true aqueous solutions in these studies is a definite disadvantage and makes the interpretation of data difficult. This is particularly true in experiments in which intravenous administration is employed, for then physical factors such as particle size may influence the results.

The physical properties of gramicidin and tyrocidine limit their application to infections in which local therapy can be employed. In this connection the absence of irritating properties even of fairly concentrated suspensions of these agents is a definite advantage over other antiseptics and may extend the scope of their application. Although the toxicity of gramicidin and tyrothricin is considerably greater than that of most agents of the sulfanilamide group, the

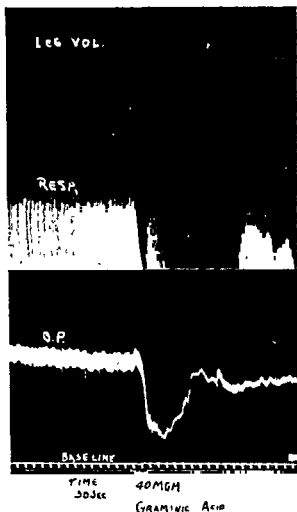


FIG. 3. EFFECT OF TYROCIDINE (GRAMINIC ACID) UPON BLOOD PRESSURE, RESPIRATION AND LEG VOLUME IN A MALE CAT 4.0 KG. BODYWEIGHT

extraordinary bacteriostatic activity of these compounds would seem to afford a large margin of safety.

Pharmacologically and toxicologically these compounds have no pronounced specific properties. They lower the arterial blood pressure, possibly because of peripheral vasodilatation, and depress the respiration. Death in acute experiments is probably due to respiratory failure, since the heart action continues for some time after the respiration has stopped. Repeated administration of toxic

doses causes degenerative changes in almost all internal organs and marked changes in the blood picture.

In view of the insolubility of these agents it is not unlikely that the effects observed are not caused by a specific pharmacodynamic action, but rather are due to non-specific physical or physico-chemical properties. It is therefore doubtful whether the toxicological results reported in this paper have a direct bearing on the clinical use of these compounds, except that application to deep lacerated wounds might approach the experimental conditions present in intravenous injection. Caution might therefore be in order whenever rapid and direct absorption of these agents into the bloodstream is likely to occur.

SUMMARY

1. The acute, peroral, intraperitoneal and intravenous toxicity of tyrothricin, gramicidin and tyrocidine was determined in mice and rats. None of the compounds was toxic when given by mouth. Upon parenteral administration all proved definitely toxic, gramicidin and tyrothricin considerably more so than tyrocidine.

2. Daily parenteral administration of 2 mgm. of gramicidin or tyrothricin per kilogram to dogs caused death within 2-8 days. During this period the dogs lost their appetite and weight and secreted excessive amounts of saliva. Their red blood counts dropped in some cases from 6,000,000 to 3,500,000 per c.mm.

3. In isolated organs as well as *in situ*, tyrocidine produced greater pharmacological changes than tyrothricin or gramicidin, possibly due to its greater solubility in water.

4. None of the drugs has a pronounced specific effect on the respiratory or circulatory system. Large single doses are usually tolerated without any marked effect, while repeated administrations even of small doses cause a fall of blood pressure and impairment of respiration. With lethal doses the respiration stops shortly before the heart.

5. Concentrations of gramicidin suspensions up to 0.5 per cent are not irritating upon instillation into the conjunctival sacs of rabbits. However, application of the dried material produces marked conjunctival irritation and a long-persisting opaqueness of the cornea. When injected subcutaneously or intradermally the preparations remain unabsorbed for a long period of time.

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STUDIES ON THE PORTAL PRESSURE EFFECTS OF DIGITALIS¹

FREDERICK P. SEDGWICK AND MAURICE B. VISSCHER

(From the Department of Physiology, University of Minnesota, Minneapolis)

Received for publication October 11, 1941

Previous investigators (1, 2, 3) have found that intravenous injection of the tinctures of strophanthus or digitalis, or the proprietary digifoline (Ciba), brings about an increase in portal venous pressure in dogs anesthetized with morphine and ether. Portal pressure elevation has also been reported (4) following very large doses of digitalis in animals under barbiturate anesthesia. Evidence has been presented which indicates that the observed rise in portal pressure is due to obstruction to venous blood flow through and from the liver, which has been called the hepatic sluice mechanism. It has been proposed (3) that the therapeutic action of digitalis depends primarily upon its effect upon the hepatic sluice mechanism.

There are reasons for doubting that the latter suggestion is correct, an important consideration being that the hepatic enlargement and ascites in cardiac decompensation in man are not increased by digitalis, as they would presumably be if digitalis produced an increase in portal pressure. Rather it is known that the reverse processes occur. Furthermore it is known (5) that in cardiac decompensation in man intravenous injection of suitable doses of pure digitalis glycosides increases cardiac output by from 25 to 40 per cent within thirty minutes after administration, as measured with the Roentgen kymograph technique. Likewise it is known (6) that doses of digitalis bodies, within the therapeutic range, increase myocardial work capacity.

Nevertheless a further study of the conditions under which a portal pressure rise can be obtained with digitalis seemed necessary because such an effect would be important even if it were solely a manifestation of toxic doses. Experiments were therefore designed to ascertain the minimum dose producing the effect. Dogs under nembutal were employed for these experiments and virtually negative results were obtained when pure glycosides were used. No consistent elevation of portal venous pressure was observed with doses in the therapeutic ranges of any crystalline glycoside used, although the tincture of digitalis and the partially purified material digifoline did exhibit an inconstant effect. Because of the difference between these findings and

¹ Aided by Technical Assistance, Project No. 65-1-71-140, Sub-Project 235 W.P.A.

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¹ Aided by Technical Assistance, Project No. 65-1-71-140, Sub-Project 235 W.P.A.

those of others, we have re-investigated the problem, with particular reference to the anesthetic agent and the digitalis body employed.

METHODS. Dogs were anesthetized either with nembutal (35-40 mgm. per kgm.) given intraperitoneally, or with morphine (20 mgm. per kgm.) subcutaneously supplemented with ether inhalation. Carotid, portal and right atrial pressures were recorded by optical manometric methods. Glass cannulae were inserted into the splenic vein directed toward the portal vein, into the right atrium through the jugular vein, and into the carotid artery. Connections were made to Gibson glass spoon manometers, as described by Kubicek, Sedgwick, and Visscher (7), by means of rigid tubing, mainly lead, connected to glass with heavy fibre tubing. The arterial manometer system was filled with 4 per cent sodium citrate solution, and the venous manometer systems were filled with isotonic saline solution containing 0.01 per cent heparin (Hynson, Westcott, and Dunning) to prevent clotting. Three-way stopcocks in the venous manometer systems permitted easy flushing under pressure with one to two cc. of the heparin-saline solution into the cannulae tips at intervals. The venous manometers were calibrated against water, and the arterial against mercury. Intravenous injections were made through a cannula in the femoral vein. Materials were injected by syringe into the rubber tubing of a burette system and flushed into the vein with 4 cc. of isotonic NaCl solution.

RESULTS. The effects of single and repeated injections of digitalis into 33 dogs have been studied in this series of experiments. The main results are summarized in tables 1 and 2. Unless otherwise noted the figures given refer to the effects of the single initial dose of the substance under study. Changes in portal and right atrial pressures of less than 1.5 cm. H₂O in either direction are recorded as zero, since fluctuations of that order occur spontaneously and are too small to be of appreciable hemodynamic significance. Changes in carotid pressure of less than 4 mm. Hg are similarly treated.

Upon inspection of table 1 it will be seen that with the exception of one instance with digitoxin (Digitaline Nativele) none of the crystalline glycosides employed caused an increase in portal venous pressure in doses from 8 to 40 per cent of the lethal, when the dogs were anesthetized with nembutal. The arterial blood pressures were ordinarily initially about 120 mm. Hg. In most instances the arterial pressures were elevated by the first digitalis injection but the further course of the arterial pressure was variable. It was noted that on repeated fractional doses, 8-12 per cent of the lethal, at 10 minute intervals, the portal pressure tended to fall until 50 to 90 per cent of the lethal amount was injected, when the portal pressure ordinarily rose. The pre-lethal rise in portal venous pressure was the most consistent finding in the series of experiments under nembutal, in confirmation of Gold and Cattell (4).

When morphine and ether were employed for anesthesia an elevation in portal pressure was found with ouabain and lanatoside C, in doses which did not produce such an effect under nembutal. However the tension rise was transient. In every case in which less than 25 per cent of the lethal dose was injected the portal venous pressure had returned to (usually below) the pre-injection level with 20 minutes.

TABLE 1

Pressure changes on administration of crystalline glycosides

NUMBER OF EXPERIMENTS*	GLYCOSIDE	DOSE*, PER CENT LETHAL	ANESTHETIC AGENT	PORTAL PRESSURE CHANGE†	ATRIAL PRESSURE CHANGE	CAROTID PRESSURE CHANGE†
				cm. H ₂ O	cm. H ₂ O	mm. Hg
1	Digitoxin	8	Nembutal	+3	0	+6
2	Digitoxin	8	Nembutal	0	0	+15
3	Digitoxin	80‡	Nembutal	-3	0	+7
1	Ouabain	8	Nembutal	0	0	+4
1	Ouabain	80‡	Nembutal	+3	0	+12
2	Ouabain	22	Morphine ether	+4	0	+12
2	Ouabain	44‡	Morphine ether	+15	0	+55
1	Lanatoside B	8	Nembutal	0	0	+4
1	Lanatoside B	70‡	Nembutal	0		-30
7	Lanatoside C	8	Nembutal	0		+11
1	Lanatoside C	23	Nembutal	0	+2	+11
9	Lanatoside C	40‡	Nembutal	0		-4
7	Lanatoside C	80‡	Nembutal	+4		-25
1	Lanatoside C	22§	Morphine ether	-3	0	+5
1	Lanatoside C	22¶	Morphine ether	+10		+55

* Except where otherwise indicated the doses shown were the first injections into individual animals.

† Mean maximal change where the number of experiments was 2 or more.

‡ Fractional doses in dogs given only 1 type of glycoside.

§ Following 22 per cent of the lethal dose of Ouabain.

¶ Following 44 per cent of the lethal dose of Ouabain.

|| Not recorded.

TABLE 2

Mean pressure changes on administration of tincture of digitalis and digifoline

NUMBER OF EXPERIMENTS	DRUG	DOSE, PER CENT LETHAL	ANESTHETIC AGENT	PORTAL PRESSURE CHANGES	ATRIAL PRESSURE CHANGES	CAROTID PRESSURE CHANGES
				cm. H ₂ O	cm. H ₂ O	mm. Hg
1	Tincture	22	Nembutal	0	0	-10
1	Tincture	45	Nembutal	+10	0	+18
2	Tincture	20	Morphine-ether	+8	0	+20
6	Digifoline	8	Nembutal	+3*	0	+4
7	Digifoline	19	Nembutal	+2†	0	+15

* 1 dog showed change less than 1 cm. H₂O.

† 4 dogs showed changes less than 1 cm. H₂O.

Table 2 presents the observed data concerning tincture of digitalis and digifoline. In marked contrast to the crystalline glycosides, these materials produce portal pressure elevation in a majority of instances, even in dogs

anesthetized with nembutal. In confirmation of previous investigators we find the tincture of digitalis produces a marked, though transient portal pressure rise when 30 per cent of the lethal dose is administered under ether.

During the course of these studies it was noted that Hynson, Westcott and Dunning heparin regularly produced a transient rise in portal pressure when injected in amount (20 mgm. per kgm., 5 units per mgm.) adequate to render blood incoagulable. Connaught heparin did not exhibit this property when comparable or larger anticoagulant doses (0.3 cc per kgm., 1000 units per cc.) were injected. Because the more active material (Connaught) had no portal pressure effect the basis of the reaction to the other commercial preparation was not investigated further. Heparinization with material from either source was not found to influence the portal pressure reaction to digitalis bodies.

DISCUSSION. The portal pressure increase which others have observed following digitalis body injection and ascribed to constriction of hepatic vessels, could exert a profound hemodynamic effect upon the heart and circulation under appropriate circumstances. However, in order to permit the conclusion that digitalis bodies generally act therapeutically through constriction of the hepatic sluice mechanism it would be necessary to have proof: (1) That all cardio-active glycosides have such an effect in therapeutic doses, and (2) that the effect persists long enough to "unload" the heart effectively.

The evidence presented in this paper is against both of these points. First, we have shown that none of four crystalline glycosides studied possesses the property of consistently elevating portal venous pressure in barbitalized dogs, under circumstances in which the tincture of digitalis and digifoline do produce such an effect. The barbiturates are not known to interfere with the therapeutic action of digitalis glycosides. Their therapeutic effects have been studied under various barbiturates (6). Further, it is known (8) that the toxicity of digitalis bodies both for cats and dogs is greater under ether than under nembutal, the difference being approximately 50 per cent. It is not impossible that the increased sensitivity to digitalis under ether is due to a greater peripheral vascular effect of digitalis bodies in toxic doses in etherized animals. It should be noted that the lethal dose of at least one digitalis glycoside, lanatoside C, for the dog is identical under nembutal anesthesia and in the unanesthetized state (8). There is, therefore, some evidence that the barbitalized animal approximates the normal unanesthetized state more closely with respect to digitalis action than does the etherized animal.

As for the second point, even if for the moment one ignores the fact that under the influence of barbiturates the effect is not seen at all with crystalline glycosides, one can consider the cases in which there is evidence of hepatic vascular constriction. Digifoline produced a rise in portal pressure with doses between 8 and 19 per cent of lethal, that is, in doses of therapeutic importance, in 8 of 13 trials. However, neither with digifoline or the tincture

under nembutal, nor with any drug under ether, was the duration of the portal pressure rise greater than 20 minutes when the dose injected was less than 25 per cent of the lethal. The average duration of the rise in portal pressure was less than 10 minutes. Furthermore the long-time trend of portal pressure is a slight decrease rather than an increase until 50 to 90 per cent of the lethal amount has been injected. It is difficult to comprehend a mechanism which, by taking a small load off the heart for 20 minutes, would set in motion processes which would rapidly reverse the phenonema of heart failure over a period of days thereafter. It is our opinion that the short duration of the rise in portal pressure renders extremely unlikely the possibility that it reflects an action (hepatic sluice closure) of primary importance to the therapeutic action of digitalis, which is characterized by the great duration of its action. It is more likely that the portal pressure effect, when it occurs, is a side reaction of the drug, of no importance to the main therapeutic effect upon the heart (6).

CONCLUSIONS

1. The effects of several digitalis bodies upon portal venous pressure have been studied in the dog in relation to type of anesthesia and dose of drug.

2. Intravenous injection of the crystalline glycosides, digitoxin, ouabain, lanatoside B and lanatoside C produced an elevation in portal venous pressure in doses from 8 to 44 per cent of lethal in only 1 instance in 23 trials, in barbitalized dogs. Under morphine and ether such an effect occurred in 5 out of 6 trials.

3. Tincture of *Digitalis purpurea* and digifoline (Ciba) produced portal pressure rises in 9 of 15 barbitalized dogs. The previously reported portal pressure rise in morphine-ether anesthetized dogs produced by tincture of digitalis has been confirmed.

4. The duration of the portal venous pressure rise produced by doses of digitalis bodies, 25 per cent or less of the lethal dose, was never greater than 20 minutes, under the conditions of these experiments.

5. It is pointed out that the facts presented concerning the elevation in portal venous pressure by cardiac glycosides are not consistent with the view that portal stasis is an essential element in the mechanism of digitalis action.

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When morphine is added to normal minced muscle *in vitro* the oxygen consumption is increased. Observations were made to determine whether a similar effect could be obtained with muscle from chronically morphinized rats. The results are presented in table 1. Added morphine produces an absolute increase in oxygen uptake which is quantitatively similar to that

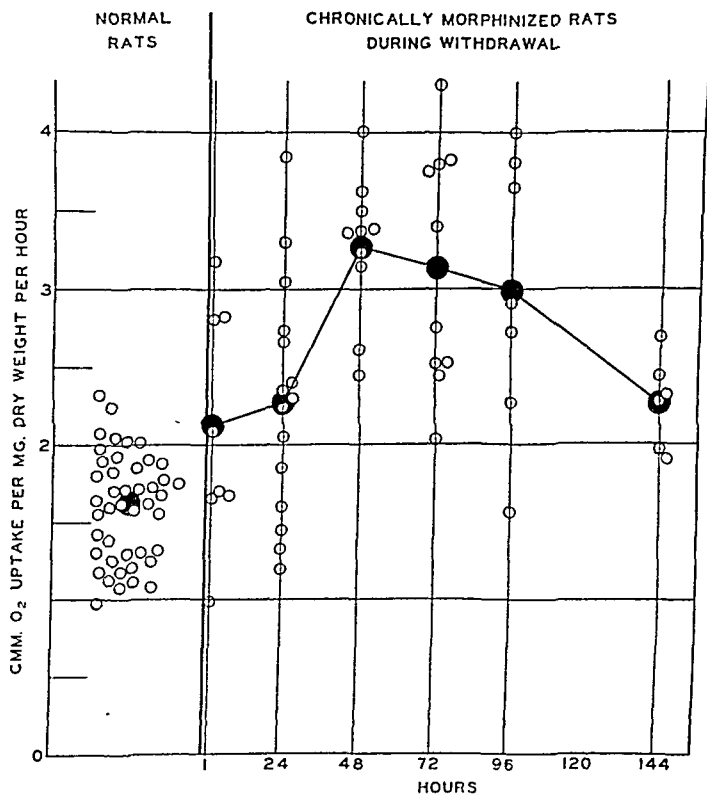


FIG. 1. OXYGEN CONSUMPTION OF MINCED SKELETAL MUSCLE OF NORMAL RATS AND CHRONICALLY MORPHINIZED RATS AT VARIOUS INTERVALS DURING WITHDRAWAL
Each unfilled circle represents Q_{O_2} on muscle from one animal

which occurs in normal muscle even though the percentage increase is smaller due to the greater Q_{O_2} of the chronically morphinized muscle.

In order to establish preliminary data which might serve as a point of departure for the subsequent identification of the oxidative systems involved during chronic morphine poisoning, the action of two commonly used inhibitors, malonate and azide, has been determined.

The addition of 0.02 *M* malonate produces about the same percentage inhibition of oxygen uptake of both chronically morphinized and normal muscle (table 2). As a result the absolute reduction of oxygen consumption is greater in chronically morphinized muscle since the initial rate of oxygen consumption is higher.

TABLE 1

Increased oxygen utilization of minced skeletal muscle from normal and chronically morphinized rats resulting from the addition of 0.12 per cent morphine

	TIME AFTER LAST INJECTION	NUMBER OF ANIMALS	MEAN Q_{O_2}	MEAN MORPHINE EFFECT
Normal		42	1.57	+0.55
Chronically morphinized	1 hour	4	2.26	+0.43
	24 hours	15	2.27	+0.46
	48 hours	6	3.03	+0.36
	72 hours	8	3.26	+0.68
	96 hours	2	2.13	+0.00
	6 days	4	2.34	+0.42
	12 days	1	1.98	+0.00

TABLE 2

Effect of malonate and azide on the oxygen consumption of minced skeletal muscle from normal and chronically morphinized rats

INHIBITOR	CONDITION OF ANIMALS	NUMBER OF ANIMALS	TIME AFTER LAST INJECTION	MEAN Q_{O_2}	MEAN INHIBITION	MEAN PERCENTAGE INHIBITION
Sodium malonate (0.02 <i>M</i>)	Normal	5		1.58	-0.70	-50
	Chronically morphinized	2	1 hour	2.25	-1.24	-55
		5	24 hours	2.19	-1.31	-60
		2	48 hours	3.35	-2.24	-66
		2	72 hours	3.76	-2.25	-60
		2	96 hours	2.14	-1.28	-60
		1	12 days	1.98	-1.28	-65
Sodium azide (0.01 <i>M</i>)	Normal	4		1.26	+0.07	+5
	Chronically morphinized	6	1 hour	2.07	-0.70	-35
		6	6 days	2.26	-0.70	-31

The oxygen uptake of a normal muscle mince is not altered significantly by the addition of 0.01 *M* sodium azide (table 2). On the other hand, this compound produces a 30 to 40 per cent reduction in the oxygen uptake of chronically morphinized muscle so that the final values for Q_{O_2} now lie within the range found for normal muscle.

A few experiments were conducted with D-140 (1-methyl-4-phenyl-piperidine carbonic acid ethyl ester),² a compound which is stated to have a spasmolytic action on smooth and skeletal muscle. In the concentration used (0.12 per cent), it reduces the oxygen uptake of both normal and chronically morphinized muscle. These results are somewhat similar to those obtained with malonate.

Barbour, Hunter and Richey (4) reported an increased hydration of the blood during morphine withdrawal and state that a similar condition probably obtains in tissues in general. Even though such were the case it would not affect the significance of the results presented here since the Q_{O_2} is expressed on a basis of dry weight. The data presented in table 3 indicate that no significant change in water content of skeletal muscle occurred in these animals during withdrawal.

TABLE 3
Water content of skeletal muscle of normal and chronically morphinized rats

	HOURS OF WITHDRAWAL	NUMBER OF ANIMALS	WATER CONTENT	
			Mean	Range
			<i>per cent</i>	<i>per cent</i>
Normal rats		42	75.8	71.9-77.7
Chronically morphinized rats	1	8	76.5	74.7-78.3
	24	15	75.2	70.8-77.7
	48	10	75.2	73.6-76.9
	72	10	75.9	74.1-76.8
	96	7	75.4	74.4-76.9
	144	6	74.6	74.0-75.3
	12 days	1	76.2	

DISCUSSION. The results presented here represent clear-cut evidence that a fundamental change in functional activity occurs in certain tissues during chronic morphine poisoning which is demonstrable even after these tissues have been isolated from their humoral and nervous connections. Whereas the full significance of this observation and its relation to addiction is not entirely apparent at the present time, it would seem to be more than a coincidence that a curve which represents the increase in oxidative metabolism of skeletal muscle of the chronically morphinized rat during withdrawal should parallel almost exactly in its time relationships one representing the intensity of the abstinence syndrome in the dog (5, 6), monkey (7, 8), and man (3).

These results support in a general way a working hypothesis which we outlined in an earlier paper (1). It is visualized that morphine exerts a sustained and reasonably uniform accelerating action on oxidations in skeletal

² Alba Pharmaceutical Company. Furnished through the courtesy of O. W. Barlow.

muscle throughout the course of addiction, but that during maintenance this effect is not detected by changes in bodily functions since the depressant action of the drug on the brain masks this peripheral effect. The specific evidence which lends the greatest support for this concept is the result obtained on chronically morphinized muscle taken one hour after the last dose of morphine. Whereas it must be admitted that no final conclusion is warranted until these results are confirmed, since the number of animals is quite small, the mean Q_{O_2} of muscle at the one-hour-period is considerably higher than that obtained on normal muscle.

It is quite obvious that the curve shown here, which represents the rate of oxidations in skeletal muscle during withdrawal, would not parallel a curve which could be drawn representing the detoxication or elimination of morphine from the tissues of the same animal over an identical time period. As a matter of fact, a major portion of the drug present in the body at the time of withdrawal would have been eliminated by the seventy-second hour, at which time the rate of oxygen uptake in chronically morphinized muscle is still near the peak of the curve. If the increase in oxygen uptake which occurs when morphine is added to normal muscle in acute experiments bears any connection to the increase in oxygen uptake above the normal level which occurs uniformly in chronically morphinized muscle during withdrawal, the exact relationship is not clarified by the foregoing facts. Nor is it easy to understand why the addition of morphine in acute experiments produces the same absolute increase in oxygen utilization in chronically morphinized as in normal muscle even though the initial Q_{O_2} of morphinized muscle is nearly twice as great. This latter observation might suggest that in both instances the excess oxygen utilization was due to the oxidation of morphine itself, a concept which we can neither prove nor entirely disprove at this time. The meager evidence which is available at this time indicates that such is not the case. It is hard to conceive of the increased Q_{O_2} of chronically morphinized muscle as being due to the oxidation of morphine already present in tissues since it increases and remains high as the morphine content of the tissues diminishes.

Stannard (9) has presented evidence which indicates that azide has a definitive inhibitory action on what he terms activity metabolism, although it does not affect resting metabolism. If this view is correct, then the increased oxygen uptake of chronically morphinized muscle must be related to activity since a concentration of azide which is without effect on normal muscle reduces the oxygen utilization of chronically morphinized muscle until the final Q_{O_2} falls into the range obtained for normal muscle.

Whereas an azide-sensitive system which is not active in normal resting muscle appears in chronically morphinized muscle, the data available indicate that the activity of the malonate-sensitive fraction of respiration is increased in proportion to the increase in total respiration. Thus malonate produces

a greater absolute inhibition in chronically morphinized than in normal muscle, yet the percentile inhibition is the same. It appears, therefore, as if the malonate-sensitive fraction of respiration is affected quantitatively but not qualitatively by chronic morphine poisoning.

SUMMARY

The rate of oxygen consumption of minced skeletal muscle from normal rats has been determined and compared with similar data from chronically morphinized rats sacrificed at 24 hour intervals during the first week of withdrawal.

The mean Q_{O_2} of chronically morphinized muscle from 56 animals calculated without regard to time of withdrawal was 61 per cent greater than the corresponding value for 44 normal rats. The rate of oxygen consumption was greater than normal even one hour after the last dose of morphine and increased rapidly during the first forty-eight hours until at this period it was double the normal level. This high rate persisted until the 96th hour, then gradually subsided but remained above the normal mean at the sixth day. A curve representing these levels of oxygen utilization during the first week of withdrawal parallels almost exactly in its time relationships one representing the intensity of the abstinence syndrome.

The addition of morphine produced an increase in oxygen uptake which was the same in chronically morphinized as in normal muscle regardless of the existing level of metabolism.

Azide, in a concentration which has no significant effect on normal muscle, abolished the increment in oxygen uptake which results from chronic morphine poisoning.

Since malonate produced the same percentage inhibition in chronically morphinized as in normal muscle, irrespective of the level of oxygen consumption, it appears as if the malonate-sensitive fraction of respiration is affected quantitatively rather than qualitatively by chronic morphine poisoning.

The authors are indebted to James K. Theisen for assistance in this study.

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EXPERIMENTAL COMPARISON OF SEVERAL ALKYL MERCURIC CHLORIDES AS "SKIN STERILIZING" AGENTS¹

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Among the antiseptics in common use, several organic aryl derivatives of mercury are prominent. The alkyl derivatives of mercury, on the other hand, have been almost completely neglected in the many published studies of antiseptics. Many of these substances are well known to chemists. That they would have bactericidal properties seemed highly probable.

A careful search of the literature revealed that apparently the only published study of the *in vivo* bactericidal action of this type of mercurial was that of Bass (1) who found that the skin-sterilizing power of *n*-propylmercuric chloride was greater than that of several other common antiseptics. The present paper describes the results of a similar careful comparison of several simple alkylmercuric chlorides. As the *n*-propyl derivative is included, these results can be compared directly with those obtained by Bass.

EXPERIMENTAL. The compounds studied were prepared in this laboratory by well-known methods: the methyl- and ethylmercuric halides from the corresponding dialkylmercury (2), the higher derivatives through the corresponding Grignard reagent (3). The products were obtained pure by two or three recrystallizations from ethanol. The test solutions were prepared by dissolving the crystalline mercurial in 95 per cent ethanol and diluting to the desired concentration. It is somewhat easier first to dissolve the substances in a small volume of acetone and then to dilute with alcohol and water.

The comparisons of the skin-sterilizing powers of these substances were made by the *in vivo* "skin-graft" technique using rabbit skin as described by Bass (1). Special care was taken to test for bacteriostatic action. The results are summarized in table 1.

DISCUSSION. The data show that all solutions of 0.0036 molar concentration in 70 per cent ethanol produced a high percentage of sterilization. The differences between these figures are not significant. The data for the more dilute solutions show that the use of 25 per cent ethanol almost eliminated the sterilization factor due to the solvent. The fact that the *per cent sterile* is about the same

¹ The funds for carrying out this work were kindly given to us by the Mallinckrodt Chemical Works.

for the 0.0036 molar (1:1000) propylmercuric chloride in the two solvents indicates that there is a considerable overlapping of the action of the solute and the 70 per cent ethanol. That the higher concentration of the solute should be more effective was to be expected; experimentally, it has been shown by Smith, Czarnetzky and Mudd (4) that the effectiveness of various mercurials against microorganisms is directly proportional to the amounts of mercury present relative to the numbers of the bacteria and/or the amounts of protein. Since in our experiments the several alkylmercuric chlorides were made up in equimolar solu-

TABLE 1

ALKYL-MERCURIC CHLORIDE	M.P., CORRECTED	SOLVENT (ETHANOL-WATER)	CONCENTRATION (MOLAR)	NUMBER OF RABBITS	MEDICATED SKIN SNIPS		STANDARD OF COMPARISON*		DIFFERENCE†
					Total number	Per cent sterile	Total number	Per cent sterile	
	°C.	per cent							
Methyl.....	174-5	25	0.0018	5	60	31.6	60	1.6	30.0
Ethyl.....	195-6	25	0.0018	5	60	33.0	60	1.6	31.4
n-Propyl.....	144-5	25	0.0018	4	48	41.7	48	0.0	41.7
n-Propyl.....		25	0.0036	7	84	84.5	84	1.2	83.3
Methyl.....		70	0.0036	6	72	91.7	72	50.0	41.7
Ethyl.....		70	0.0036	6	72	83.3	72	47.2	36.1
n-Propyl.....		70	0.0036	4	48	81.3	48	27.1	54.2
n-Butyl.....	128-9	70	0.0036	4	48	93.7	48	47.9	45.8
n-Amyl.....	127-8	70	0.0036	7	84	97.6	84	46.4	51.2
n-Heptyl.....	120-1	70	0.0036	4	47	87.2	47	34.2	53.0

* In each case the standard of comparison was the same as the solvent for the antiseptic.

† The figures in the column headed "Difference" represent the per cent sterile of the medicated skin snips, minus the per cent sterile of the skin snips treated with the control solutions.

From each rabbit three pieces of untreated skin were cultured and invariably gave good growths.

All "cultures" which remained clear after 48 hours incubation were inoculated with from 50 to 1000 organisms obtained from an untreated skin culture. All these flasks developed good growths. Thus bacteriostasis was negligible.

tions, any differences in effectiveness would have been attributable to differences in penetrating power.

Preliminary tests with several other alkylmercury compounds were carried out. A number of the simple *R-Hg-Br* derivatives showed definite bactericidal action, but as their solubilities were lower than those of the chlorides—and they seemed to offer no advantages—they were not studied further. A solution of 0.034 per cent diethylmercury in 70 per cent ethanol was ineffective.

In consideration of the possible clinical use of these compounds for pre-operative skin sterilization, preliminary tests were performed with the 1:1000 solution of propylmercuric chloride. In our experience the normal unbroken human skin

has shown no ill effects from the several times repeated daily application of the solution. No effort to measure directly the antiseptic's effectiveness on human skin has been made.

Favorable results have been obtained in the treatment of several cases of trichophytosis. Occasionally a blister has occurred, probably due to sensitivity of the skin in the infected area, or to the building of a high concentration of the mercurial by repeating the applications as the solvent evaporated.

SUMMARY

The compounds $R\text{-}\overset{\cdot\cdot}{\text{Hg}}\text{-Cl}$, in which R is methyl, ethyl, n -propyl, n -butyl, n -amyl and n -heptyl, have been tested as skin disinfectants, using the *in vivo* "skin-graft" technique on rabbit skin. The 0.0036 molar solutions in 70 per cent ethanol all gave a high percentage of sterile skin snips. If the 0.0036 molar (1:1000) solution of n -propylmercuric chloride is taken as a standard, and the above results thus compared with those of Bass (1), it is found that solutions of the simple alkylmercuric chlorides are as effective as any and more effective than many of the commonly used antiseptic solutions when compared by this method.

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IN VITRO ACTION OF SULFONAMIDES ON LYMPHOGRANULOMA VENEREUM VIRUS

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Treatment with sulfonamide derivatives of four virus diseases has been reported; these are trachoma (1), inclusion blennorrhea (2), a (distemper-like) ferret virus disease (3), and lymphogranuloma venereum or lymphopathia. The first observations on the experimental treatment of lymphogranuloma venereum virus infection were those with prontosil (4) in infections of mice and guinea pigs. Sulfanilamide was found to retard the development of the virus in mice (5). This report was later substantiated by the successful treatment of mice with sulfapyridine (6). McKee, Rake, Greep and van Dyke (7) concluded that sulfapyridine and sulfathiazole had an appreciable effect upon the virus of lymphogranuloma venereum in mice.

That accessory growth factors are rendered inutilizable is one of the theories suggested for the mode of action of the sulfonamide compounds (8, 9). Substances antagonistic to the action of sulfanilamide were found in bacterial extracts (10, 11, 12). At about the same time Woods (13) proved that para aminobenzoic acid had a similar inhibiting effect which was believed to be due to interference with the metabolism of the organisms (14). Additional inhibitors appearing or existing in the tissues and fluids of the body were demonstrated by MacLeod (15). From clinical observations on lymphogranuloma venereum and on blennorrhea, Stein (16) and Thygeson (2) were of the opinion that the drug acted directly upon the virus. In view of these facts it seemed advisable to study experimentally the action of the sulfonamides on lymphogranuloma virus.

By *in vitro* experiments we have attempted to determine the virucidal or inhibitory effect of chemotherapeutic substances in direct contact with the virus of lymphogranuloma venereum and to compare the efficacy of certain sulfonamide compounds. Findlay and MacCallum, 1938 (17), attempted similar experiments using decreasing dilutions of the virus with constant amounts of sulfanilamide, without success.

MATERIAL AND METHODS. *Virus:* A strain of the virus of lymphogranuloma venereum, B367, was obtained through the kindness of Dr. S. A. Wykoff, from the Lederle Laboratories, Inc. This was inoculated in successive passages intracerebrally until the strain was consistently pathogenic. The animals showed marked loss of weight, ruffled hair, humped back, conjunctivitis and paralysis; death resulted in five to ten days depending on the

amount of virus injected. In order to determine the most appropriate dilution for our experiments, 0.03 cc. of dilutions 1:20, 1:200 and 1:500 were inoculated intracerebrally in mice. After these virus dilutions had been in the refrigerator over night, other groups of mice were inoculated. Mice receiving 1:20 dilutions of the infected mouse brain showed symptoms within three to four days, death occurring within five days; there was no apparent change in virulence of the virus upon standing for 24 hours at ice box temperature. The mice inoculated with the dilution 1:200 showed acute symptoms within 3 to 5 days, death occurring within 7 days. There was a slight decrease in virulence after standing for 24 hours at ice box temperature. With the dilution of the virus 1:500 the incubation period and the severity of the symptoms varied. Our regular passage mice were subsequently inoculated with dilution 1:20. After four or five days, when definite symptoms appeared, they were killed and a dilution of 1:200 brain emulsion was used for the experiment. Routine cultures were made to detect bacterial contamination.

Periodically, Frei antigens (18) were made from brain emulsions of mice showing acute symptoms. These antigens were tested on patients in parallel with antigen made from the brain of normal mice and a Frei antigen obtained from the Lederle Laboratories, Inc. Our virus antigen and the Lederle antigen gave parallel positive tests. The normal mouse brain antigen was negative.

Mice: White mice, obtained from the Carworth Farms, Inc., 15-17 grams in weight, were used for the experiments. To guard against a latent virus such as that of Theiler (19) and Traub (20), in our stock mice, the following safeguards were employed. At various intervals 0.03 cc. of a mouse brain emulsion of a normal mouse was inoculated intracerebrally into three mice. Passages were made from these mice on the seventh day and carried for four passages. At no time was there any evidence of virus disease; the passage mice remained normal.

Sulfonamides: The drugs employed were sodium sulfanilyl sulfanilate, sulfaguanidine, sulfathiazole and sulfadiazine.

Method: To determine changes in the incubation period, the duration of symptoms and the percentage survival, mice were inoculated with virus exposed to the drugs for different periods. Control mice were inoculated with untreated virus. Solutions of 100, 150, and 200 mgm. in 100 cc. of hot distilled water were made of each drug. Sulfadiazine, the least soluble, was tested out only in 100 mgm. to 100 cc. of hot distilled water.¹ After cooling to room temperature, 2 cc. of each of these solutions was placed in sterile test tubes. To each was added 0.1 cc. of 1:10 dilution of an infected mouse brain emulsion, giving a 1:200 dilution of the virus. The mixtures were shaken and placed in the incubator at 37°C. for one hour, then kept at room temperature for one hour. Following this two hour period, 0.03 cc. was inoculated intracerebrally into 6 to 10 mice. The remainder of the mixtures were kept in the refrigerator for twenty-four hours. Thus the inoculation periods were 2, 6 and 24 hours of virus and drug contact.

Control mice were inoculated with the same dilutions of virus in distilled water, incubated and kept under the same conditions and inoculated at the same time. A control group of mice was inoculated with the drug dilutions without virus. The mice were observed for a month.

In all experiments the technic provided for the comparison of the four drugs in the same experiment, on the same day, using the same virus passage for the dilutions so that there was no variation in virulence, incubation period or temperature. At various intervals, dead mice were autopsied. Passages of the brain emulsion into other mice were made to

¹ After cooling to room temperature the concentration, as measured by the Marshall method, was 200 mg. % for sodium sulfanilyl sulfanilate, 190 mg. % for sulfathiazole, 135 mg. % for sulfaguanidine and 30 mg. % for sulfadiazine. At the end of 24 hours at ice box temperature, crystals of the drugs precipitated out and the concentrations were 200 mg. % for the sodium sulfanilyl sulfanilate, 65 mg. % for sulfathiazole, 60 mg. % for sulfaguanidine and 9.7 mg. % for sulfadiazine.

determine whether virus of lymphogranuloma venereum was present and was the only cause of death.

EXPERIMENTS. Sodium sulfanilyl sulfanilate: Six experiments were performed with this drug. Table 1 shows that the incubation period is somewhat longer in the mice inoculated with the drug-treated virus than in the control mice. A wider range is observed in a comparison of the average duration of illness, which was more than doubled after the two hour contact period and more than tripled after 24 hours of contact. These observations indicate that the virulence of the virus was attenuated by the drug. The 25 per cent survival of the drug-treated mice with no survivals in the controls supports the view that the virus was definitely attenuated but not completely destroyed because all the experimental mice showed acute symptoms of the disease with slight ameliora-

TABLE 1

2, 6 and 24 hours. Sodium sulfanilyl sulfanilate-exposed virus*

CONTACT TIME <i>hours</i>	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
2	49	20	6 4	7 3	25 0
6	41	19	7 5	8 3	29 14
24	45	16	9 7	10 3	49 18

* The incubation period was from the day of inoculation to the day symptoms of disease appeared. The duration of symptoms is from the day of appearance of symptoms to either death or recovery.

tion in those that survived during the observation period of 30 days. The symptoms did not entirely disappear.

Sulfaguanidine: Five experiments were performed with this drug. The effect of sulfaguanidine is very similar to that of sodium sulfanilyl sulfanilate. A similar increase is observed in the incubation period and the average duration of illness of the drug-treated virus mice when compared with the control mice.

Sulfathiazole: Five experiments were made with sulfathiazole. There is a definite increase in the average duration of illness and in the percentage survival, 43 per cent (table 3). The recovered mice showed symptoms of the disease with gradual amelioration.

Sulfadiazine: Four experiments were performed with sulfadiazine. Attenuation of the virus was observed here also. There was an increased incubation period and the drug-treated virus was lengthened slightly, ultimately disappearing after contact and very

slightly more with longer contact. None of the control mice, inoculated with the drug solutions without virus, showed any untoward effects from the drugs; they remained normal and gained weight.

DISCUSSION. The effect of the drugs upon the virus is shown by the prolongation of the average incubation and duration of symptoms of mice inoculated with virus exposed to the drugs for various periods as compared with the control mice. Tables 1, 2, 3 and 4 show that following the 2-hour contact period, the incubation period for the control mice was 4 days as compared

TABLE 2
2, 6 and 24 hours. *Sulfaguanidine-exposed virus*

CONTACT TIME <i>hours</i>	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
2	32	16	5 4	5 3	15 0
6	30	16	6 3	6 3	20 18
24	23	18	8 6	11 3	43 25

TABLE 3
2, 6 and 24 hours. *Sulfathiazole-exposed virus*

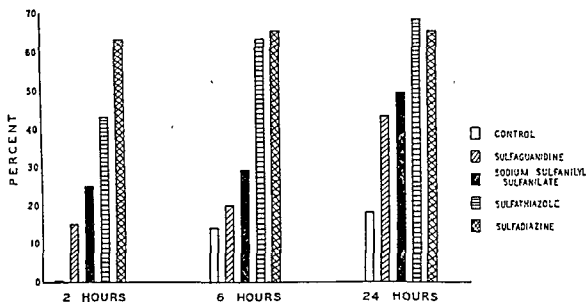
CONTACT TIME <i>hours</i>	NUMBER OF MICE		AVERAGE DAYS INCUBATION	AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control			
2	28	16	7 4	10 4	43 0
6	30	16	7 3	14 3	63 18
24	23	12	10 6	12 4	68 25

with 5 to 8 days for the drug treated mice. Similarly, it is found that following a 6-hour period the average incubation for the control mice was 4 days while that of the drug treated mice was 6 to 10 days. Following a 24 hour period, the average incubation for the control mice increased to 7 days, that for the mice inoculated with the drug treated virus was 8 to 12 days. The increase in the incubation period following a 24 hour contact is partly due to the slight loss in virulence of the virus, evidenced by the increase in the incubation time for the control mice. Animals inoculated with virus treated with

sulfaguanidine or sodium sulfanilyl sulfanilate only showed marked prolongation of acute symptoms within 24 hour contact of the virus with these drugs (see tables 1 and 2). The average duration of symptoms in the control mice was 3 days; the corresponding figure for the other mice was 10 to 11 days. Animals inoculated with the virus treated with sulfathiazole and sulfadiazine showed, in most cases, slight symptoms which were markedly prolonged after

TABLE 4
2, 6 and 24 hours. Sulfadiazine-exposed virus

CONTACT TIME <i>Hours</i>	NUMBER OF MICE		AVERAGE DAYS INCUBATION		AVERAGE DAYS DURATION OF SYMPTOMS	PERCENTAGE OF SURVIVALS
	Experimental	Control				
2	22	12	8	4	12	63
6	26	12	10	4	12	65
24	18	9	12	7	13	66
					4	0



GRAPH 1

the 2 hour contact (tables 3 and 4). While the average duration of the control mice was 4 days, that of the other mice was prolonged to 10 to 12 days.

Measured by the percentage of survivals of the mice inoculated with the drug-treated virus (Graph I) sulfaguanidine is the least effective, resulting in 15 and 20 per cent survivals after the 2-hour and 6-hour contact periods. Similarly, sodium sulfanilyl sulfanilate results in slight effect, 25 and 29 per cent after the first two contact periods. But after the longer period of contact, 24 hours, the

percentage of survivals is increased to 43 and 49 per cent. From these data it appears that sulfaguanidine and sodium sulfanilyl sulfanilate are effective on either a less virulent phase of the virus, as may be surmised from the slight loss of virulence after 24-hour standing, or when in contact for a longer period of time. However, when we deducted from the percentage of survivors after twenty-four hours' contact the percentage of survivors in the control, the same percentage of survival was found, as after 2 hours of contact (tables 1 and 2). Accordingly, the effect is probably produced mostly by the shorter contact, with little change when contact is prolonged.

The same condition was observed in the experiments with the sulfathiazole-treated virus. Deducting the percentage of the control survivals, 25 per cent, from the survivals in the mice inoculated with the drug-treated virus, 68 per cent, after 24 hours of contact, an average survival of 43 per cent resulted (table 3). This is the same as after two hours of contact (Graph I). There were more survivals with sulfathiazole than with either sodium sulfanilyl sulfanilate or with sulfaguanidine. The most effective drug was sulfadiazine,

TABLE 5
Comparison of amount of drug used and survival after 2 hours

DRUG	AMOUNT OF DRUG IN 2 CC. SOL.	PER CENT SURVIVAL
	mgm.	
Sulfadiazine.....	0.06	63
Sulfathiazole.....	0.38	43
Sodium sulfanilyl sulfanilate.....	0.4	25
Sulfaguanidine.....	0.27	15

The dilution of virus was the same in each instance.

which gave a 63 per cent survival after two hours of contact with little change thereafter (Graph I).

Taking into consideration the amount of the drugs which was contained in the 2 cc. of the saturated solution (note 2) used in the experiments, the smallest amount, 0.06 mgm., was contained in the sulfadiazine solution which gave the highest per cent of survival (table 5). Sulfathiazole solution contained slightly less drug (0.2 mgm.) than the solution of sodium sulfanilyl sulfanilate. The former resulted in a 43 per cent survival while the latter resulted in 25 per cent survival. Sulfaguanidine solution, containing 0.27 mgm., was the least effective drug resulting in a 15 per cent survival.

CONCLUSION

Direct *in vitro* contact of sulfonamide compounds with the virus of lymphogranuloma venereum reduces its virulence when subsequently tested in mice. There was no instance of virucidal action but only virus attenuation or inhibition. Sulfadiazine and sulfathiazole are most efficient in this order. Sodium sulfanilyl sulfanilate and sulfaguanidine are less efficient.

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THE URICOSURIC EFFECTS OF CERTAIN POLYHYDRIC ALCOHOLS AND SACCHARIDES¹

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In the pursuit of another problem, we had occasion to examine sorbitol in hypertonic solution as a diuretic. Our initial experiments were made with a 50 per cent solution injected intravenously. This solution not only increased the urine volume but also increased markedly the output of uric acid and allantoin. These findings led us to study the effects of other polyhydric alcohols and sugars in a similar fashion; in addition, it seemed desirable to compare the effects of these substances with mercurial diuretics which also increase the output of uric acid (1).

Female dogs with exteriorized ureters were used for these experiments. In this way we were able to collect the urine in hourly periods without manipulation and with the dog standing in a normal position. Although the urine was collected from each kidney and analyzed as such, since the total excretion per hour from the individual kidneys was equal we combined the hourly figures in order to simplify presentation of material. Results are expressed in cubic centimeters or milligrams per kilogram except as otherwise indicated on the charts. One experiment consists of three periods of one hour each. The method of preparing these animals and of collecting the urine has been described in detail elsewhere (2).

The dogs were fed at four o'clock in the afternoon on a standard diet of 150 to 500 grams of meat according to the size of the animal. Two grams of salt were added to the daily diet. Water was allowed ad libitum. All experiments were done in the morning. All the drugs were injected intravenously. Water in the water diuresis experiments was given by stomach tube. The strength of the solutions varied with the solubility of the substance used. In the case of dulcitol, we were able to give a super-saturated solution (20 per cent) by maintaining the solution at body temperature until injection. All results are shown in the accompanying charts.

Controls were of two kinds—without any drug, and after the injection of 15 cc. of 0.9 per cent sodium chloride solution. The injections used did not exceed this volume except in the case of raffinose in which a few injections of 30 cc. volume were made. Injection of 30 cc. normal salt solution produced no diuresis in one experiment. It will be seen (fig. 1), that the various constituents remain highly constant under the conditions of this experiment. There is a slight tendency for the three urinary constituents with which we are concerned to decrease in succeeding hours and averages show a distinct decrease. The chart is so plotted that the statistical validity of averages can be seen at a glance. There is a tendency for the urine volume to vary more in the first hour than subsequently and this

¹ We are indebted to the Abbott Company for the Sorbitol and Raffinose used and to the Atlas Powder Company for the Dulcitol and Mannitol.

may be explained by the possibility of the animal having had water just before it was taken out of the cage. However, the differences that are to be discussed are sufficiently large so that this variation does not matter. The excretion was the same from the two kidneys. It will be noted that the uric acid excretion never exceeded 0.09 mgm. per kilogram. Only rarely did the urine volume exceed 1.5 cc. per kilogram.

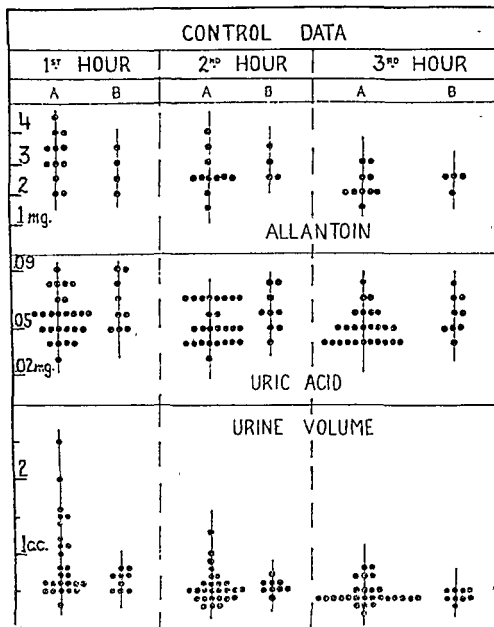


FIG. 1. CONTROL DATA. PLOTTED IN MGm. OR CC./KGM.

Column A is without any injection and B is after the intravenous injection of 15 cc. normal salt solution.

Sorbitol solution (50 per cent) causes nearly a three-fold increase in uric acid excretion in the first hour (fig. 2). During this same period, 15 cc. of 50 per cent sucrose solution doubles the average uric acid excretion; smaller increases are produced by 50 per cent fructose solution and 20 per cent glycerine. Sorbitol in 20 per cent solution is followed by a less marked but none the less definite

increase in uric acid, whereas 10 per cent sucrose and 10 per cent or smaller concentrations of sorbitol produce less change in uric acid excretion.

After the first hour the action of 50 per cent sorbitol persists significantly though slightly. The action of fructose, glycerine, and smaller quantities of sorbitol and sucrose does not continue beyond the first hour. Fifteen cubic centimeters of 10 per cent raffinose produces a very slight increase in uric acid excretion in the second and third hours. This effect is more pronounced with 30 cc. of 10 per cent raffinose and leads us to suspect that were it possible to give larger doses, a comparable but delayed rise would result. Fifteen cubic centimeters of 50 per cent dextrose, xylose, maltose, galactose, 20 per cent dulcitol

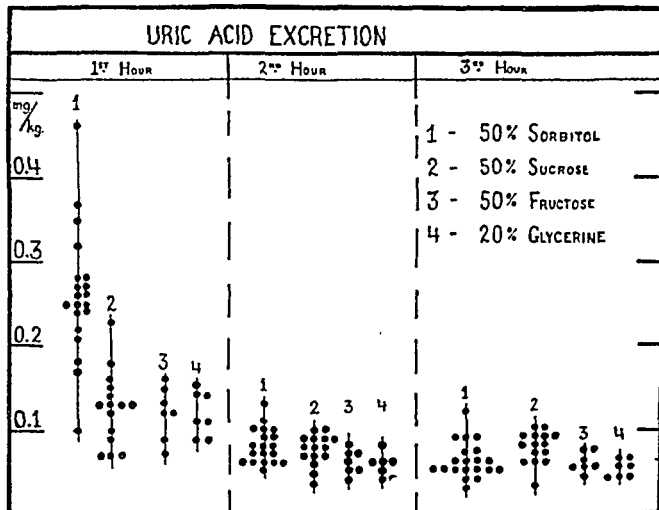


FIG. 2. PROMPT EFFECT OF URICOSURIC SUGARS. SHOWS AMOUNT OF URIC ACID EXCRETED IN THE THREE HOURS AFTER INJECTION OF THE SUGAR

and 40 per cent mannitol have no effect on the uric acid excretion for the three hours following their injection.

Water, when given by mouth in quantities of 100 to 300 cc., causes a diuresis which lasts well into the second hour and in some experiments, into the third hour (fig. 3). The other substances studied produced varying degrees of diuresis though sucrose, sorbitol and xylose were the most efficient in this respect. It is noteworthy that xylose is possibly the most effective diuretic studied though it has no effect on uric acid excretion. In lower concentrations these substances were less diuretic and concentrations as low as 10 per cent were ineffective.

We did not analyze for allantoin those urines containing sugars which on heating with ammoniacal copper solutions hydrolyzed and reduced the copper. Sorbitol, glycerine and mannitol, however, do not reduce the copper solution. We found that the excretion of allantoin was greatly increased by 50 per cent

sorbitol, slightly-increased by 20 per cent glycerine, and unaffected by smaller quantities of sorbitol and mannitol.

The output of urea, total nitrogen and chloride was always proportional to the urinary volume. Some of these sugars reduce the alkaline picrate causing increased color. Therefore, although creatinine was measured on all samples, we can draw no conclusion from the results.

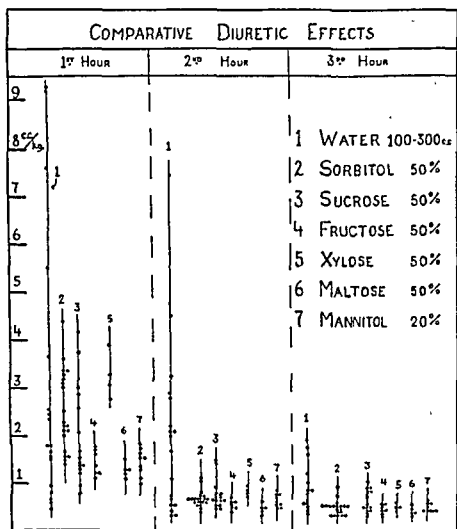


FIG. 3. DIURETIC EFFECTS TO BE COMPARED WITH CONTROLS—FIGURE 1. WATER GIVEN BY STOMACH TUBE, OTHER SUBSTANCES BY INTRAVENOUS INJECTION IN DOSES OF 15 CC. OF THE CONCENTRATION INDICATED

It has been found that 18 hours after eating, the uric acid excretion is solely endogenous (3, 4). Consequently, in all our experiments, we are probably dealing with endogenous uric acid.

The uric acid excretion is augmented by the injection of sorbitol, sucrose, fructose and glycerine. Sorbitol seems to be the most active in this respect in comparison to sucrose and fructose. We thought it advisable not to use glycerine in comparable concentrations because of possible toxicity; therefore, we cannot compare the intensity of its action with the others.

It seemed possible that the increased excretion was a result of the diuresis

causing a washout of uric acid. On comparing the output of uric acid following the injection of these substances with the excretion during water diuresis of equal or greater degree, we found no change in the latter and a large increase in the former.

Further evidence that the increase is not a result of the diuresis is that we have been able to produce an increase in uric acid by a 20 per cent solution of sorbitol

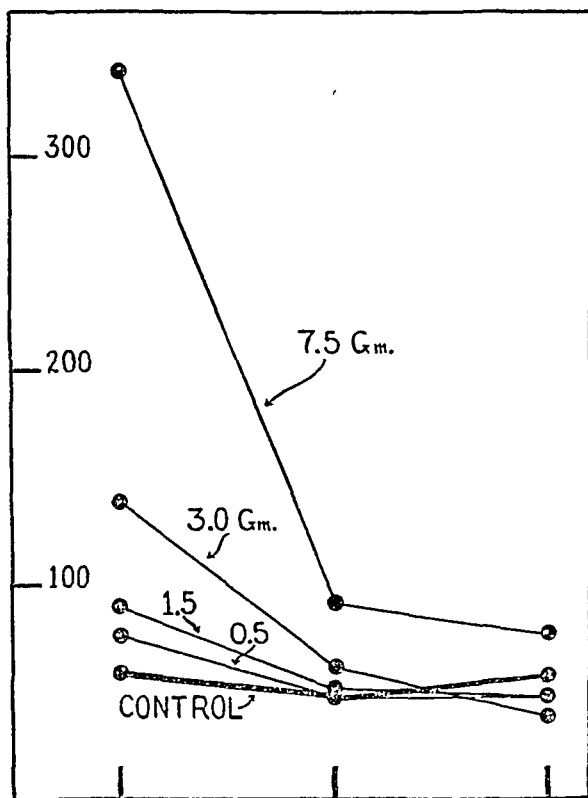


FIG. 4. AVERAGE URIC ACID EXCRETION AFTER DIFFERENT DOSES OF SORBITOL—TIME IN HOURS
Ordinates plotted in mgm./kgm. per kidney

which produces only a slight change in volume. Smaller concentrations of sorbitol and sucrose which are not diuretic do produce a small increase in uric acid. Since the increase in uric acid appears to be proportional to the dose it is likely that this is a direct effect of the drug (fig. 4).

Simultaneous injections of sorbitol, sucrose, fructose or glycerine with 0.5 mgm. ergotamine or 1 mgm. atropine cause no change in the increased excretion

of uric acid. Denervation of the kidneys produces no change in the action of these sugars. Mares, (3), Smetanka (5), Mendel and Stehle (6), and Abl (7), all found increased excretion of uric acid after the ingestion of various substances among which were sucrose and glycerine. These observers agree that the increase in uric acid excretion could be explained as the result of the activity of the digestive glands. We find that an increased excretion of uric acid can be obtained by intravenous injection of these two substances. Although we cannot say that the activity of the digestive glands following ingestion of sucrose and glycerine is not a cause of an increase in uric acid excretion, we can say that the activity of the digestive glands is not essential to the increase in uric acid in the case of sorbitol, sucrose, fructose and glycerine. In man, Thomas and Imas (8) have found an increased excretion of uric acid after the ingestion of pentoses; however, Madders and McCance (9) were not able to verify these results.

There seems to be a similarity in the structure of those substances which cause this increased output of uric acid. In all cases, there are two CH_2OH groupings, one on each end of the carbon chain. Substances without this characteristic do not affect the uric acid excretion. Both mannitol and dulcitol, which have two CH_2OH groups so placed and are isomers of sorbitol, do not increase uric acid. From this it is apparent that the optical configuration plays a role in the pharmacologic activity perhaps similar to that seen in various alkaloids in which one optical isomer is active and the other inert.

We can find no direct explanation for the variations in the diuretic responses to these substances. It seems evident that the uricosuric action is not dependent upon the diuresis. Indeed, a few short-interval experiments indicate that the peak of the uric acid excretion comes after the diuresis has begun to subside and that an elevated uric acid excretion is maintained until the urinary volume has returned to normal (fig. 5).

We are unable to explain the increase in allantoin excretion produced by sorbitol and glycerine except to point out that it supports the idea that these substances exert a definite action on the purine metabolism quite apart from their effects as diuretics.

It is evident from the foregoing that an increase in uric acid and allantoin excretion is produced by the intravenous injection of certain sugars and alcohols. Substances having this effect have, in every case, the CH_2OH group at both ends of the chain. So far, we have studied trihydric and hexahydric alcohols and certain saccharides containing this configuration. Other related substances do not affect purine excretion. This increase is not related to the "washing out" effect described by Hawk (10). It should be noted that some of these substances produce an increase in uric acid excretion when given by mouth. Mendel and other observers sought to connect this uricosuria with activity of the digestive glands. It is obvious that the prompt effects seen on intravenous injection cannot involve activity of the digestive glands. The recognized relationship between the liver and purine metabolism makes one suspect that this effect is connected with this organ. The mechanism is not at present clear. Renal denervation has no effect. Injections of ergotamine and atropine suf-

ficient to block the sympathetic and parasympathetic do not change the effect. Large doses of posterior pituitary extract likewise do not alter the result. Experiments of one dog with an Eck fistula failed to show any change in the reactions to these substances, though of course the initial level of uric acid excretion was higher. It is evident that various substances which might serve to change the uricosuric or diuretic reaction of these carbohydrates are ineffective. We must conclude that the uricosuric action is a function of their chemical structure mod-

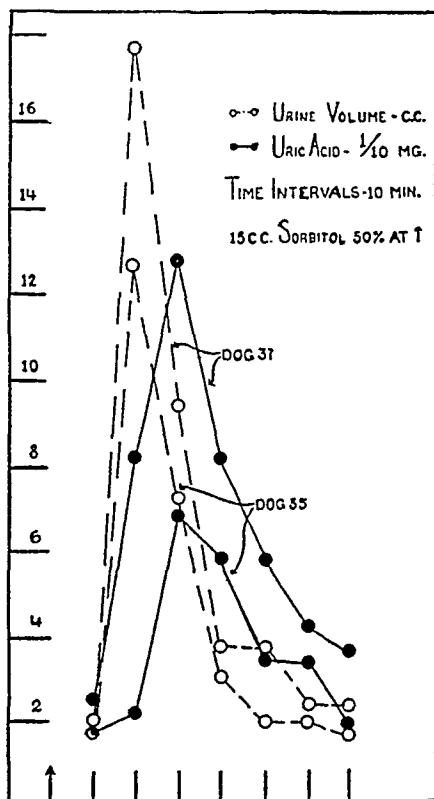


FIG. 5. RELATION BETWEEN THE DIURETIC AND URICOSURIC EFFECT OF SORBITOL

ified by the optical configuration, and is distinct from the diuretic effect which is presumably due to the physico-chemical effects of non-assimilable substances in hypertonic solution.

The observation that salyrgan diuresis is accompanied by an increased uric acid excretion (1), prompted us to use this drug to compare with these carbohydrates in the hope of throwing more light on the mechanism of the uricosuric action. While the diuretic action of salyrgan is comparable to that produced

by the sugars; the uricosuric action of the former in dogs is only trifling as compared with the latter. It was also noted that diuresis produced by salyrgan seemed greater in dogs with one kidney denervated, which suggests that the mechanism of diuretic and uricosuric action of sugars and salyrgan was different, since the action of the sugars was unaffected by denervation, atropine or ergotamine.

SUMMARY

1. The effects of intravenous injections of hypertonic solutions of certain polyhydric alcohols and saccharides have been studied in dogs with exteriorized ureters without anesthesia.
2. These drugs produce not only a diuresis but also a marked increase in uric acid and allantoin excretion.
3. All substances causing the increase in purine excretion have the CH_2OH grouping at both ends of the chain.
4. This is not accomplished through a nervous mechanism.
5. It is unlikely that the hypertonicity of the solution per se is the cause of uric acid mobilization, since some hypertonic solutions do not produce this result.

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A STUDY OF THE TOXIC PROPERTIES OF TUNG NUTS

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The present investigation was undertaken for the purpose of determining what part of the tung nut contains the active toxic principle, in the hope that this substance could be isolated and identified. The toxic nature of tung nuts has been known for a long time, but few instances of experimental study of this property have been reported. Gardner (2) relates an instance of the ingestion of tung nuts by humans. The violent purgative effect which resulted prompted him to study the physiological action of tung oil on rabbits and dogs, in the hope that materials of therapeutic use might be discovered. He found, however, that relatively large doses had only a mild laxative effect. The oil produced no irritation of the intestinal mucosa and no interference with normal functions was observed, although the experiments were continued over a long period of time. His investigation was not extended to other parts of the tung fruit. Carratala (1) has given an excellent description of the symptoms which follow the ingestion of the fruit of the tung tree (*Aleurites fordii*), in describing the poisoning of five workers who had eaten the nuts. None ate more than three of the nuts, but soon showed violent epigastralgia, typical colic pains, bilious vomiting, burning thirst, and profuse diarrhea. As to nervous reactions, there were noted paresthesia, formication, cramps in the legs and arms, nausea, and exhaustion. We have been given an accurate account of the symptoms following the eating of only one tung nut by a student of the University, who mistook it for a Brazil nut. An hour later he felt dizzy and weak and had severe pains in the stomach. Violent vomiting, accompanied by diarrhea, set in immediately, and recurred at short intervals. Attempts to satisfy an unusual thirst seemed to aggravate this condition, which lasted for 6 or 7 hours, after which he was very weak. On the following day he had a headache throughout the morning, but there was no pain. By the third day he had completely recovered.

Godden (3) carried on extensive feeding experiments with tung meal, obtained by solvent extraction, using rats, pigs, and dairy cattle as test animals. The meal which he used was steamed for 40 minutes and dried in air before being used. With these animals a purgative effect was observed, and they lost weight and condition on rations containing meal up to 25 per cent. None of the animals died from the effect of the meal although rats were kept on the ration for 21 days. Godden concluded that the material contained "some substance which makes it unpalatable, and some irritant which has a harmful effect on the mucous membrane of the intestines."

Several instances of dermatitis caused by vapors of heated tung oil have been reported (4, 5), and attributed to the toxic nature of tung nuts.

EXPERIMENTAL. The procedure adopted in this work involves a step-wise separation of various constituents of the tung nut with solvents and the testing of the physiological action of the various components so obtained. The object of the separation was the isolation and study of the toxic principle of the nut. The white rat was used as a test animal. The fractions isolated were usually administered in the form of a ration consisting of a mixture of the material and ground dog chow, which was moistened with cane syrup. The resulting dough could be worked into balls of the desired weight, in which form it could be easily fed.

Physiological action. A. Tung nut kernels. Each of 11 rats was given 15 grams of a ration containing 50 per cent of tung nut kernels. The rats ate this material readily. After three hours a purgative effect was observed in seven cases. After 18 hours this effect was apparent in all of the animals. On the second day 5 of the animals were replaced on their regular diet and they returned to normal in a few days. The other 6 rats were kept on the ration containing tung nut kernels, but after the first day they did not eat the preparation so readily. They became sluggish and apparently very much weakened. On the fourth day one of the animals died. Two died on the seventh day and two more on the eleventh. Only one rat survived to the thirteenth day, when the experiment was discontinued.

B. Tung oil, separated from (A) by extraction with petroleum ether. A group of 5 rats was placed on a ration containing 24 per cent of tung oil. The rats ate this preparation readily enough, but remained perfectly normal although they were kept on the ration for 5 days. There was no sign of the purgative effect observed as when the tung nut kernel was fed.

C. Tung meal, separated from (A) by extraction with petroleum ether. Each of 5 rats was given 15 grams of a ration containing 19.5 per cent of the oil-free meal. After 14 hours, all of the rats showed definite signs of a purgative effect, and after 38 hours they seemed sluggish and weak. At the end of 62 hours two of the animals were found dead. At this time each of the other three animals was given an additional 5 grams of the food mixture, and at the end of 86 hours none survived.

D. Tung meal, after extraction of (C) with alcohol. A group of 5 rats was given daily 10 grams each of a mixture containing 19.5 per cent of tung meal (D). After five days the animals remained perfectly normal and the experiment was discontinued.

E. Material, obtained from alcohol extract of (C). About 130 grams of oil-free tung meal (C) was extracted for 10 hours in a continuous extractor with 95 per cent alcohol. The meal remained at 150-160°F. during this time. Upon evaporation of the alcohol a pasty reddish-brown residue was obtained, which became lighter in color and definitely crystalline in appearance when washed with petroleum ether. The yield was 3.85 grams. This solid material was administered orally, in water solution, in doses of 0.3 grams (equivalent to 10.1 grams oil-free

meal) to each of 5 rats. The animals were kept on their regular ration and observed for 24 hours. No physiological effect was observed. The doses were repeated, but again no effect was observed.

The extracted tung meal (*D*) and the material (*E*) were mixed in the same proportions in which they existed in the original oil-free meal (*C*), and tested in the feeding experiments as above. The experiment was continued for 5 days, but none of the symptoms of tung nut poisoning was observed.

F. Tung meal, after various heat treatments. A sample of oil-free tung meal (*C*), after heating in a hot air oven at 195°–205°F. for 4 hours, was made up into a ration containing 19.5 per cent of this material, which was fed to a group of 5 rats at the rate of 5 grams each daily. On the second day a slight purgative effect was noted. The experiment was continued for six days, but none of the rats died or showed the sluggishness observed when unheated meal was fed.

The experiment was repeated with meal which had been heated in a hot air oven at 212°–247°F. for 15 hours, with the results that no signs of a purgative effect were observed during the six days that the experiment was continued.

A quantity of oil-free tung meal (*C*) was heated at 230°F. with steam in a pressure cooker for two hours, dried, and tested in the feeding experiments as above. Five rats were given 10 grams each per day for 5 days, but no toxic symptoms appeared.

G. Press cake. A ration containing 27 per cent of tung press cake was given to each of 5 rats in 10 gram portions daily. (The press cake is the product obtained from the commercial pressing process. The ground kernels are heated with steam before being pressed.) The rats ate most of the food given them, and after three days, no harmful effects were observed.

DISCUSSION. In this investigation it is shown that the decorticated kernels of tung nuts are toxic to rats. When finely ground kernels are included in the food of white rats the animals quickly develop signs of a purgative effect. After some time the animals grow sluggish and weak and finally die.

If the oil is removed from the kernels by extraction with petroleum ether (Skelly Solv "A") before mixing with the food, the toxicity persists. In this case symptoms of poisoning follow more quickly. In an experiment conducted on five rats with oil-free meal, all of the animals died within 86 hours after the first feeding. The extracted oil, after removal of solvent, had no effect upon rats, either when mixed with the food or when administered orally by means of a pipette in doses as large as 1.5 cc.

After the toxicity of the oil-free kernels was established, the material was extracted with alcohol for ten hours, during which time the temperature of the material was 150°–160°F. The residual meal was not toxic, nor was the solid material obtained upon evaporation of the alcoholic extract. The latter material was added to the former in the same ratio that they existed in the meal before extraction, but this mixture also had no physiological effect. The long heating in the presence of alcohol apparently either decomposed the toxic substance or destroyed some enzyme necessary for its formation.

Our experiments show that heating with steam at 230°F. for two hours renders

the oil-free meal non-toxic to rats. Heating in a dry condition apparently requires a longer time to render the material non-toxic, for when the dry meal was heated in a hot air oven at 195°-205°F. for four hours and given to rats, the animals developed slight symptoms of the purgative effect produced by unheated meal but did not grow sluggish or weak. The dry meal was rendered completely non-toxic by heating for fifteen hours at 212°-247°F. in an oven.

In view of the effectiveness of steam upon the highly toxic oil-free meal, the non-toxic nature of the press cake investigated here is especially of interest, as the press cake was obtained from ground kernels which had been steamed before pressing in the commercial plant. The results thus far obtained indicate that a perfectly non-toxic material may be readily obtained as a product of the tung oil industry.

The authors are indebted to the late Dr. Charles E. Coates for helpful suggestions, and to Mr. D. T. Cushing of the Great Southern Lumber Co., Bogalusa, La., who provided samples of tung nuts, press cake, and tung oil.

SUMMARY

The toxic principle of tung nuts is not present in tung oil, but is found in tung meal. A method by which this principle may be destroyed is described. An attempt to isolate the toxic material was unsuccessful.

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THE DELAY IN ONSET OF ACTION OF INTRAVENOUSLY INJECTED ANESTHETICS¹

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The delay that occurs between the intravenous introduction of certain narcotics and the full development of their action has attracted relatively little attention and has not been satisfactorily explained. The first narcotic found to show this lag was α -*d*-glucochloralose. Soon after the discovery of the narcotic activity of this drug, the lag was clearly described (1). In the other important class of narcotics having slow onset of action, the barbituric acids, this property was not so promptly recognized. For several years after the introduction of barbital into medicine nearly all of the laboratory and clinical studies were made with the acid form given by mouth. The first reference that I have found to the delay in onset of narcotic action after intravenous injection of a barbituric acid is that of Impens (2), who described it for phenobarbital in 1912. His reference to the lag is casual but unequivocal. Other early references to the slow onset of action of barbituric acids are those of Tiffeneau (3) (barbital, dial, ethyl-allyl-barbituric acid) and of Starkenstein (4) (barbital). Of the other drugs tabulated in this paper as having lags, some have previously been reported to have this property. Others have not. Some have even been described as having immediate onsets (e.g., nostal and nirvanol (5)).

PART 1. *Relation between dose and lag.* For the investigation of the relationship between dose and lag, phenobarbital and α -*d*-glucochloralose were chosen as representative of the two principal classes of narcotics having lags. Phenobarbital was selected as the representative of the barbituric acids because, of all those with long lags, it gives the quietest anesthesia. The time at which anesthesia is reached is, for that reason, more easily and accurately determined. Moreover, the duration of phenobarbital anesthesia is very long, and the form of the dose-lag relation should be relatively little affected by the inactivation of the drug. Less exactly determined data for barbital indicate that the dose-lag relation is of a similar form to that of phenobarbital. At corresponding doses, the lags are about 1.8 times as great as those of phenobarbital.

¹ This work was supported by a grant from Mallinckrodt Chemical Works. Some of the drugs used were kindly supplied by the following firms: Hoffmann-LaRoche, Eli Lilly & Co., Riedel-de Haen, Sandoz Chemical Works, Sharp and Dohme, and Winthrop Chemical Co.

METHODS. Both drugs were given intravenously to male white mice. Phenobarbital was given as a freshly prepared solution of the sodium salt. The concentrations of the solutions were made proportional to the dose, so that all mice receiving the same drug received the same volume of solution per gram of body weight (phenobarbital, 0.017 cc.; chloralose, 0.03 cc.). The injection of each of the 5 lower doses of phenobarbital occupied $\frac{1}{4}$ minute (1 minute for the highest dose); each dose of chloralose, 2 minutes.

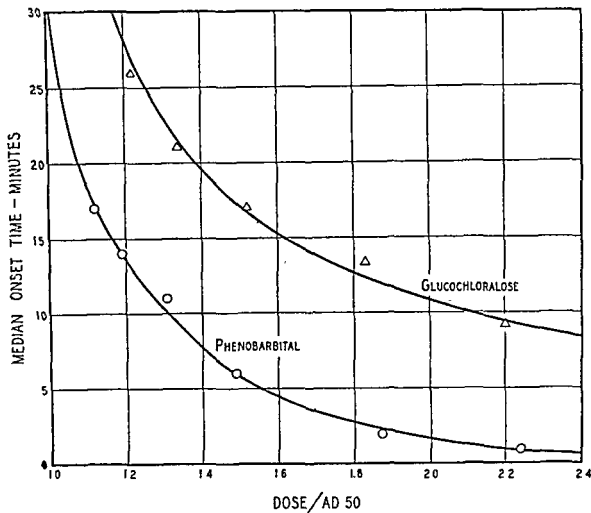


FIG. 1. RELATION BETWEEN DOSAGE AND TIME OF ONSET OF ANESTHESIA

For each point 25 mice were used. Ordinate—median time between end of injection and onset of anesthesia. Abscissa—ratio of dose to the previously determined median anesthetic dose of the drug (chloralose, 0.082 mgm. per gram; phenobarbital, 0.134 mgm. per gram). The curve fitted to the chloralose data is $y = 36 \log_{10} \frac{x}{x-1}$; that fitted to the phenobarbital data is $y = 19e^{-2.5(x-1)}(x-1)^{-0.1}$.

The 150 mice receiving the 6 doses of phenobarbital represented in figure 1 were assigned in groups of 6 at random to the 6 doses. The 6 mice were all dosed on the same day. A similar procedure was used for the 5 doses of chloralose.

The median anesthetic doses, which enter into the expression of dosage used in figure 1, were determined by the same method used for all of the other drugs. (See Part 2.)

The criteria of anesthesia were the same as those described in Part 2.

The results are shown in figure 1. Median rather than mean values of time are plotted because it was thought that this would give a picture of the dose-

time relationship more representative of that which would be found if it could be determined for an individual mouse.²

To draw any very definite conclusions from the shapes of the curves would be hazardous.³ Time-concentration curves for much simpler biological systems have yielded very little information as to the physico-chemical processes involved in the delays. (For a review of this subject, see Clark (7).) The shapes of the two curves are sufficiently different to indicate the need of some caution in assuming that the cause of the lag is the same for the chloraloses as for the barbituric acids. However, either is compatible with the hypothesis that diffusion is an important factor in the delay.

PART 2. Comparison of the lags of various anesthetics. As was shown in Part 1, the lag is to a great extent dependent on the dose. For this reason, if the lags of different drugs are to be compared, it is important that doses be used that give comparable depths of anesthesia. In an attempt to fulfill this condition, I have determined the median anesthetic dose of each drug and measured the lag at a dose 1.25 times this value. This ratio gives a dose that proved to be sufficient to anesthetize every mouse with every drug tried.

Drugs were chosen to represent a large variety of chemical classes of narcotics. The majority of those tabulated are derivatives of barbituric acid. The large number of readily available drugs of this group show a wide range of lags. This series affords a good opportunity of studying the relation between structure and lag.

* Suppose the dose-lag relation of a population of mice to be represented by the family of curves, $y = f(x, a)$, the parameter, a , determining the position of the asymptote (anesthetic dose). Then the relation between dose and the arithmetic mean of the lags shown by two or more of the curves will not in general be of the form $y = f(x, a)$.

The shape of the curves in figure 1 suggests that if the anesthetic doses of a population of mice were normally distributed, the distribution of the lags measured at any dose would not be normal but positively skewed (mean greater than mode). The asymmetry would be more pronounced at lower doses. Calculation of g_1 (Fisher (6), p. 78) shows that the distribution of the times for the lowest dose of phenobarbital departs significantly from normality (positive skewness). For the other 5 doses of phenobarbital, the asymmetry is not significant. On none of the 5 doses of chloralose is there significant asymmetry. Of all the other drugs (Part 2) for which the lags were measured at 1.25 times the median dose, only barbital, nostal, and sandoptal have significantly asymmetrical distributions of lags (all positively skewed).

* No particular significance should be attached to the equations used for drawing the curves through the observed points, even though the chloralose equation is of a type that expresses the course of several familiar physical and chemical processes (e.g., the time required for a given amount of solute to diffuse across a thin membrane in accordance with Fick's law). These are the simplest equations giving asymptotes at $x = 1$ and $y = 0$ that were found to give a reasonably good fit. (Since the duration of anesthesia is finite with respect to the lag in onset, a curve showing two values of y for each value of x , with a tangent at $x = 1$, would be a still better representation of reality.) Since a number of different time-concentration relations have been found to fit equilateral hyperbolas and since certain diffusion processes are also represented by such curves, I have tried to fit equilateral hyperbolas to the present data. The data can be fitted rather roughly (considerably less closely than by the curves shown) by these hyperbolas: phenobarbital, $y(x - 1) = 2.7$; chloralose, $(y - 5)(x - 1) = 5.5$.

METHODS. *Source of drugs.* Those drugs that were not obtainable in a good state of purity either commercially or from the laboratories of the manufacturers were synthesized in this laboratory or isolated from commercial mixtures and purified.

Administration of drugs. All drugs were given intravenously to male white mice. The concentrations were usually made such that a mouse would receive about 0.015 cc. of solution per gram body weight, but the low solubility and low activity of some drugs necessitated giving considerably larger volumes of solution. All of the barbituric acids were given as freshly prepared solutions of the sodium salts.

Criteria of anesthesia. A mouse was considered anesthetized if it was unable to gain and maintain the standing position after stimulation by repeated pinching of the tail.

Determination of the median anesthetic dose (AD 50). For the drugs of tables 1 and 3, two doses were found, one of which would anesthetize between 24 and 50 per cent of the mice, the other between 50 and 76 per cent. Twenty-five mice were used on each of these two doses. The median dose was estimated by interpolation from these two groups on the assumption that the relation between dose and proportion anesthetized is linear over this range.⁴

For all of the other drugs (none of which had a lag), only 12 animals were used on each of the two doses. The precision of these doses is consequently less than that of those determined from the larger groups.

The doses of the barbituric acids and hydantoins are expressed in terms of the acid form.

The series of doses shown in the tables were determined over a period of several years and in all seasons. The question of seasonal and other such variations in sensitivity to drugs must be considered in comparing the activities of these drugs.

Measurement of lag. For the drugs of tables 1 and 3 a dose 1.25 times the previously determined median anesthetic dose was given. The injection time was usually $\frac{1}{2}$ minute. Longer injection times were necessary for a few drugs (e.g., 2 minutes for the chloraloses). The time was noted at which the mouse reached the level of anesthesia described above. Times were measured from the end of the injection. Twenty-five mice were used for each drug, the mean time being tabulated (see footnote ³). The lag was always measured within a few days of the determination of the anesthetic dose. The mice used came from the same group from which those used for the dose determination had come. This procedure probably nullified to some extent the effects of the above-mentioned variations on the measured lags.

If in the determination of the median anesthetic dose every mouse that became anesthetized did so within a few seconds of the end of the injection, the use of the higher dose was omitted and the drug classified as having no lag. The onset of action of these drugs may not be quite instantaneous and the drugs may differ slightly among themselves in their rapidity of onset. However, their lags are too short to be determined with any degree of significance by the method described above.

All of the anesthetics that I have found to have any measurable delay in onset of action are to be found in tables 1 and 3. It is to be noted that all of these drugs fall into two chemical classes: the chloraloses, and the 5,5-disubstituted derivatives of barbituric acid and the closely related compound, hydantoin.⁵ Although I have tested a considerable number of anesthetics belonging to other

⁴ While this assumption is of course not true, it can lead to no serious error in this range. The assumption of more complex relations, which doubtless are better approximations, leads to values of the median dose that differ from those calculated here by amounts so small as to be of no significance in this study.

⁵ The 5,5-disubstituted derivatives of another compound related to barbituric acid, oxazolidinedione, are reported by Stoughton and Baxter (8) to have no lag.

chemical groups, no others have been found to have lags.⁶ In table 4 are listed a few of the anesthetics that have immediate onset of action. This list is chosen to contain representatives of diverse chemical classes and to show a wide range of anesthetic doses.

Examination of table 1 suggests that among the 5,5-disubstituted barbituric acids there is a tendency for the longer lags to be found in the less active drugs. That this association is statistically significant can be demonstrated by the treatment of figure 2. Here are plotted the anesthetic doses and lags of the 18

TABLE 1

Anesthetic doses and lags of eighteen 5,5-disubstituted derivatives of barbituric acid

The tabulated lag is the mean time of onset of anesthesia in 25 mice given a dose 1.25 X the AD 50.

BARBITURIC ACID	MOL. WT.	AD 50		MEAN LAG IN ONSET
		mgm. per gram	moles X 10 ⁻³ per gram	minutes
5-ethyl-5-ethyl (Barbital).....	184.1	0.234	127	22.0
-5-phenyl (Phenobarbital).....	232.1	0.134	58	12.3
-5-isopropyl (Ipral).....	198.1	0.110	56	9.2
-5-(1-cyclohexenyl) (Phanodorn).....	236.1	0.110	47	0.9
-5-butyl (Neonal).....	212.1	0.076	36	1.6
-5-hexyl (Ortal).....	240.2	0.078	32	0
-5-isoamyl (Amytal).....	226.2	0.054	24	0.2
-5-(1-methyl-1-butenyl) (Delvinal).....	224.1	0.047	21	1.7
-5-(1-methylbutyl) (Pentobarbital).....	226.2	0.033	15	0.1
5-allyl-5-allyl (Dial).....	208.1	0.073	35	12.8
-5-isopropyl (Alurate).....	210.1	0.058	28	12.4
-5-isobutyl (Sandoptal).....	224.1	0.063	28	3.4
-5-(2-cyclopentenyl) (Cyclopal).....	234.1	0.060	26	1.6
-5-(1-methylbutyl) (Seconal).....	238.2	0.028	12	0.1
5-(2-bromoallyl)-5-isopropyl (Nostal).....	289.0	0.071	25	9.5
-5-sec-butyl (Pernoston).....	303.1	0.049	16	2.7
-5-(1-methylbutyl) (Sigmodal).....	317.1	0.040	13	0.2
5-methyl-5-(1-cyclohexenyl).....	222.1	0.206	93	8.9

barbituric acids of table 1. Lines are drawn through the mean values of dose and lag, dividing the plot into 4 quadrants. If anesthetic dose and lag were independent properties, the ratio of the number of drugs falling in Quadrant II to the number in Quadrant III should equal the ratio of the number in

⁶ So far as I am aware, the only other drug that has been reported to have slow onset of action after intravenous administration is 3,3-diethyl-2,4-diketo-piperidine (itself similar in structure to barbital). Foster (9) found a lag with this drug in rabbits. In rabbits I also find a brief lag, but not in mice.

Quadrant I to the number in Quadrant IV. The observed ratios differ significantly, indicating that anesthetic dose and lag are associated properties.⁷

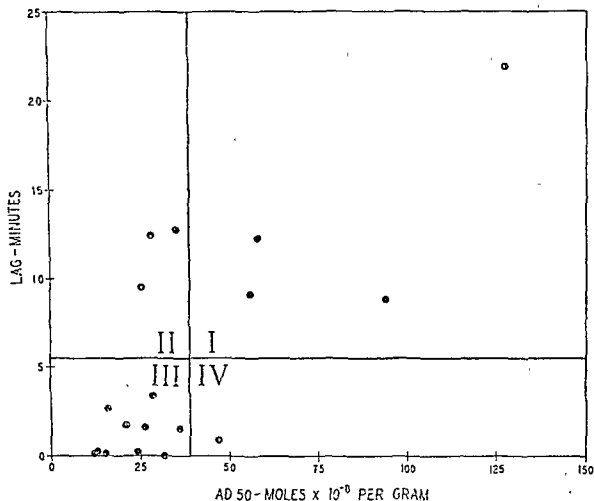


FIG. 2. THE EIGHTEEN 5,5-DISUBSTITUTED BARBITURIC ACIDS OF TABLE I
The perpendicular lines are drawn through the mean values of dose and lag

No *N*-substituted barbituric acid or thiobarbituric acid was found that had a lag (table 2). Although a *N*-substituted compound is more active than the

⁷ Of the 18 drugs, 7 have lags above the mean, 11 below the mean. If dose and lag were independent properties, the 13 drugs having anesthetic doses below the mean should contain the same proportion of high lags and low lags as 13 drugs selected at random from the 18. If 13 drugs are selected at random from the 18, the probability that these 13 will contain 10 or more below-the-mean lags is

$$\frac{5! 13!}{18!} \left(\frac{7! \times 11}{4! 3!} + \frac{7!}{5! 2!} \right) = 0.047$$

This probability is so low that the hypothesis of independence cannot reasonably be accepted.

This test of independence involves no assumptions regarding the form of distribution of lags or doses, nor regarding the form of the relationship between dose and lag. Because the distributions of the doses and the lags cannot be regarded as normal (the distribution of these 18 doses shows significant positive skewness), the correlation coefficient cannot be applied here.

corresponding unsubstituted compound and a thiobarbituric acid more active than its oxygen analogue, it is to be noted that some of the drugs in table 2 are less active than the rapidly acting compounds of table 1.

DISCUSSION. The earliest attempt to explain the slow onset of action of a narcotic is that of Overton (10) (1901). He found that the narcosis produced by chloralose develops slowly in tadpoles and disappears slowly when they are transferred to fresh water. Chloralose was the only strong indifferent narcotic known by Overton to behave in this manner. By plasmolytic experiments, a method entirely independent of the appearance of narcosis, he demonstrated that chloralose enters plant cells slowly. In this respect, also, it differed from the other indifferent narcotics. Overton thought that the rapidity of entrance of a compound into a cell is determined by its solubility in the lipoids of the cell

TABLE 2

1,5,5-trisubstituted derivatives of barbituric acid and 5,5-disubstituted derivatives of thiobarbituric acid

The onset of action of all of these drugs is almost immediate.

BARBITURIC ACID	MOL. WT.	AD 50	
		mgm. per gram	moles $\times 10^{-4}$ per gram
1-methyl-barbital.....	198.1	0.070	35
1-ethyl-barbital.....	212.1	0.110	52
1-propyl-barbital.....	226.2	0.038	17
1-allyl-barbital.....	224.1	0.055	25
1-butyl-barbital.....	240.2	0.060	25
1-methyl-phenobarbital (Mebaral).....	246.1	0.050	20
1-methyl-alurate (Narconumal).....	224.1	0.032	14
1,5-dimethyl-5-(1-cyclohexenyl)-barbituric acid (Evipal).....	236.1	0.029	12
5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid (Pentothal).....	242.2	0.012	5
5,5-diethyl-2-thiobarbituric acid.....	200.2	0.140	70

membrane relative to its solubility in water. He evidently assumed that narcosis is produced only after the penetration of the drug into the cell.

Of a different nature is the suggestion offered by Klimesch (5) to account for the slowly developing action of certain barbituric acids. Klimesch attributed the differences in rapidity of onset of action of barbituric acids to the differences in degree of hydrolysis of their sodium salts. The greater the proportion of the drug present in the blood and tissues in the form of the lipoid-soluble acid, the more rapid the onset of action was assumed to be. This idea was reaffirmed in a subsequent paper by Starkenstein and Klimesch (11). Bush (12) has pointed out that the method used by Klimesch to estimate the degree of hydrolysis of the salts is not valid. Bush determined the dissociation constants of five barbituric acids and, failing to find these values correlated with the lags, showed that the hypothesis of Klimesch is not acceptable. Barbital and amytal, two drugs that have lags differing in the extreme, were found to have equal

dissociation constants. The independence of dissociation constant and lag is corroborated by the study of a longer list of dissociation constants recently published by Clowes *et al.* (13). Fourteen of the barbituric acids of table 1 are to be found in this list. Among these fourteen, there is no evidence of association between pK' and lag.^{*}

Owing to the infinite complexity of the cell, the possibilities of describing its functions in terms of familiar physico-chemical models are severely limited. However, since the greater part of the time must be accounted for by processes the course of which is greatly affected by small changes in chemical structure or physical properties, it is possible that a study of the association between lag and the properties of the drugs may yield a clue at least to the general nature of the principal factor in the delay.

The first question that arises concerning the lag is whether it is the time required for the drug to arrive at its site of action or whether it is a delay in the biological response of the cell after the primary action of the drug is complete. Among the 5,5-disubstituted barbituric acids listed in table 1, drugs that differ among themselves relatively little in chemical structure, there are to be found lags of all magnitudes. It is difficult to conceive of drugs so closely related chemically differing among themselves in their sites or mechanisms of action. On the other hand, the differences in physical properties are such as might be expected greatly to influence the speed of passage from the blood to the site of action. Therefore, at least so far as the barbituric acids are concerned, the hypothesis that the delay is in the arrival of the drug at its site of action appears the more attractive.

The significant association between narcotic activity and rapidity of onset demonstrated here may throw some light on the nature of the process by which the drug reaches its site of action. Such an association would result if this mechanism were dependent on some physical property of the drug that is also associated with narcotic activity. Among the barbituric acids as well as other narcotics, there is a general tendency for the more active drugs to have a higher solubility in certain organic solvents relative to water. This solubility relationship is often thought of in terms of the distribution coefficient between olive oil and water, not because of any unique physiological significance of this particular coefficient but because it was the one used by the early advocates of the lipoid theory of narcosis. Let it be assumed that the lag in onset of narcosis is due to diffusion of the drug from an aqueous phase into or across a non-aqueous phase. Then if the more active drugs had higher partition coefficients between the non-aqueous solvent and water, they would pass more rapidly into and across the non-aqueous phase, and the observed association between anesthetic dose and lag would result.

The fact that there is also a marked parallelism between the oil/water distri-

^{*} If these data are treated by the method of figure 2, pK' (from Clowes *et al.*) being plotted as abscissa and lag (from table 1) as ordinate, the number of drugs falling in quadrants I, II, III, and IV are, respectively, 3, 3, 4, 4. These numbers do not differ from those expected from the hypothesis of independence.

bution coefficients of many organic substances and their speeds of penetration through cell membranes suggests that the non-aqueous phase assumed above might be the lipid layer of the cell membrane.⁹ If some of the assumptions considered above were combined, the hypothesis would assume this more specific form:

The delay in onset of narcotic action is the time required for the drug to penetrate into or through the cell membrane, the more rapid penetration of the more active drugs being due to their greater relative solubility in the lipoids of the membrane.

TABLE 3

Anesthetics other than barbituric acids having slow onset of action

The lag was measured in the same way as for the barbituric acids of table 1.

DRUG	MOL. WT.	AD 50		MEAN LAG IN ONSET
		mgm. per gram	moles \times 10^{-4} per gram	minutes
5-ethyl-5-phenyl-hydantoin (Nirvanol).....	204.1	0.161	79	14.5
α -d-glucochloralose.....	309.5	0.082	27	24.4
α -d-arabinochloralose.....	279.4	0.410	147	15.6
α -l-arabinochloralose.....	279.4	0.199	71	9.8

TABLE 4

Some anesthetics other than barbituric acids having immediate onset of action

DRUG	MOL. WT.	AD 50	
		mgm. per gram	moles $\times 10^{-4}$ per gram
Methanol.....	32.0	7.0	22,000
Chloral hydrate.....	165.4	0.27	163
Ethyl acetate.....	88.1	0.27	306
Ethyl carbamate.....	89.1	1.12	1,250
Paraldehyde.....	132.1	0.73	553
Acetone.....	58.0	2.4	4,140
Sulfonal.....	228.3	0.50	219

This hypothesis is essentially an application of Overton's theory of cellular permeability. However, it is to be noted that it has been assumed above that the amount of drug passing from the original aqueous phase is at least roughly proportional to the observed anesthetic dose. This assumption is not consistent with the requirements of the lipid theory of *narcosis*, at least as set forth by Meyer and Hemmi (15).

The hypothesis mentioned above is inadequate to explain the unequal rates of onset of narcosis following injection of the enantiomorphous arabinochloraloses,

⁹ Among the 9 barbituric acids of table 1 for which olive oil/water distribution coefficients have been published (14) there is a significant association between distribution coefficient and lag.

for the physical properties of these compounds are identical. It is conceivable that the chloraloses differ entirely from the barbituric acids in their site and mechanism of action. The lag might still be considered a delay in the arrival of the drug at its site of action if it were assumed that the drug penetrates not through simple diffusion in accordance with Fick's law, but by some process that limits the rate of transfer. Thus the larger quantity of a less active drug would require a longer time to reach the site of action.

No reason is apparent why the lag should be found in such a limited number of chemical classes of narcotics. Reference to table 4 indicates that it cannot be solely a matter of narcotic activity or of molecular dimensions or of solubility. One must assume that those chemical groups in which lags are found narcotize at a different site or through a different mechanism or arrive at their site of action through a different mechanism from other narcotics.

SUMMARY

A delay occurs between the intravenous introduction of certain anesthetics and the full development of their action. The only drugs that I have found to have this property are chloraloses and 5,5-disubstituted derivatives of barbituric acid and hydantoin. The lags of a number of these drugs have been measured at doses designed to give comparable depths of anesthesia.

Among the eighteen 5,5-disubstituted barbituric acids studied, it has been demonstrated that anesthetic dose and lag are associated properties, the more active drugs tending to have more rapid onset of action.

This association might be explained if it were assumed that the delay is the time required for the drug to penetrate into or through the cell membrane, the more rapid penetration of the more active drugs being due to their greater relative solubility in the lipoids of the membrane.

This hypothesis is inadequate to explain the unequal rates of onset of the antipodal arabinochloraloses.

No reason is apparent for the fact that the property of slow onset of action is limited to those chemical classes named.

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SOME PROPERTIES OF FICIN

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During recent years a number of investigators have reported studies on the properties of the enzyme contained in the milky sap of the fig. Such studies have been prompted by its enzymatic properties as well as by its historic status as a Spanish-American anthelmintic. An excellent review of its history and bibliography is given by Asenjo (1). The purpose of this paper is to present the results of further investigation along lines suggested by its use as an anthelmintic.¹

The partially purified enzyme, dried to constant weight in a vacuum desiccator at room temperature showed, by Kjeldahl, a total nitrogen content of 11.1 per cent, corresponding to 69.6 per cent protein. Its average ratio of free amino nitrogen to total nitrogen, the former determined by the Van Slyke method, ranged from 13 to 14 per cent in different samples, which places it approximately in the proteose class. In its proteolytic properties it resembles papain in that it has an optimum pH of about 5, as shown by Robbins (2). This pH was therefore used for all determinations of proteolytic activity. We found that it rapidly hydrolyzed proteins such as egg albumin, gelatin, etc. at room temperature, but carried them to the proteose-peptone stage with a free amino nitrogen total nitrogen ratio of about 25 per cent. When added to commercial proteose-peptone mixtures little or no further hydrolysis was observed. Thus in the degree of hydrolysis accomplished it resembles gastric pepsin.

The thermo-lability of ficin was first reported by Robbins (3), who used its effect on *Ascaris* as the criterion. We have confirmed his results using hydrolysis of egg albumin, measured by the production of non-precipitable (by trichloroacetic acid) nitrogen as the criterion. Complete inactivation resulted from one hour's heating at from 70° to 75°C. The enzyme is not poisoned in any measurable degree by the presence of chloroform. Treatment of a water solution with ketene under conditions which should acetylate only free amino groups yielded a product with about two-thirds the enzymatic activity of the untreated sample.

The fairly homogeneous character of our enzyme preparation is indicated by the results of dialysis through a collodion membrane. As is the case with most proteose-peptone mixtures, a portion dialyzes easily through such a membrane but the molecular complexity of the dialysate, as indicated by the free amino

¹ The work was carried on with partially purified samples of the enzyme in powdered form furnished us by the kindness of Merck and Company, Incorporated.

nitrogen total nitrogen ratio, does not differ markedly from that of the residue. Furthermore, experiments of this type have shown enzymic activity on the part of the dialysate proportional to the total protein content of the solution.

One of the remarkable properties of this enzyme, no doubt responsible for its continued medicinal use, is that of digesting living intestinal helminths. Such ability obviously does not accord with our conception of the mechanism of action of proteolytic enzymes but a member of experiments with freshly collected, live, pig *Ascaris* provide ample confirmation of the results first reported by Robbins (3). The helminths are rapidly attacked by the enzyme with the production of extensive lesions of the body wall. During the course of this digestion the power of locomotion of the worms leaves no doubt that they remain alive for some time. However, long-continued action by the enzyme solution produces death and almost complete disintegration of the worms, with marked increase in the nitrogen content of the solution in which they are immersed.

Site of action. In an attempt to determine whether the action of ficin on live *ascaris* is on the exterior body surface or through the alimentary tract of the worms, the following experiment was carried out: Freshly collected live *Ascaris* were divided into three groups of five worms each. *Group I* was immersed in 200 cc. 0.1 per cent ficin made in Locke's solution; *Group II* was immersed in the same volume of Locke's solution without the ficin. *Group III* was a duplicate of Group I in the ficin solution but, whereas the worms of Group I had their alimentary tract closed by ligation as described by Brown (4), those of Group III were left unligated. All three groups were incubated at 37°C. and observed at intervals up to 150 minutes. At the end of that period the worms of Groups I and III, while retaining the power of locomotion, had a badly blistered cuticular surface whereas Group II (controls) showed no damage. However, no difference could be observed between Groups I and III; since ligation of the alimentary tract was without result, the action of the enzyme was purely on the exterior surface of the worms. Moreover, the increase in the nitrogen content of the solution as digestion proceeded was practically identical in Groups I and III in spite of the inability of the enzyme to gain entrance to the intestinal tract of the worms of Group I. A similar experiment in physiological saline solution gave the same results.

Fecal excretion. The clinical use of crude preparations of ficin for the treatment of Trichuriasis has been described by Brown (5), and recommended by various writers as reviewed by Asenjo (1). The degree of success recorded for crude ficin preparations is the more remarkable because the habitat of *Trichuris* is the cecum. Thus the necessary site of action is so far down the intestinal tract as to subject the enzyme, evidently of protein nature, to the digestive action of the proteolytic enzymes of the stomach and small intestine. The literature contains little or no exact information as to the relative abilities of different proteolytic enzymes to destroy one another but one must assume, in this case, that some of the ficin passes unaffected through the areas of tryptic and ereptic digestion and arrives at the cecum with, at least its anthelmintic if not its proteolytic properties unimpaired. In this ability, it appears to be in sharp contrast to the

animal's ordinary digestive enzymes which are almost entirely absent from normal feces. It seemed worth while, therefore, to test whether or not the proteolytic activity of doses of ficin can survive to the point of excretion in the feces. It is well known that feces normally show little or no tryptic activity.

The question of fecal survival of ficin was tested as follows: Doses of partially purified and dried ficin (used in the above-described experiments) were administered in water solution to two dogs at levels of 0.1 gram and 0.5 gram respectively per kilo body weight. Twenty-four hour collections of feces were made, both for several days before administration of the enzyme and for a longer period afterwards. The dogs were kept throughout the experiments on an ordinary animal house diet. The 24-hour samples of feces, varying considerably in weight, were each macerated in water; the resulting mixture was filtered and washed into 500 cc. volumetric flasks and made up to volume; 25 cc. aliquots of these solutions were used for testing proteolytic activity.

Standardization was effected by running a series of tests with varying amounts of the same sample of ficin as that administered to the dogs in 25 cc. water. This was added to

TABLE 1

Absolute increase in percentage of free amino- to total nitrogen effected by varying amounts of ficin in 25 cc. water solution added to 200 cc. 2% gelatin solution. Room temperature

WEIGHT OF FICIN IN 25 CC. SAMPLE	ABSOLUTE RISE IN PERCENTAGE OF FREE AMINO- TO TOTAL NITROGEN	
	30 minutes	60 minutes
grams		
0	0	0
0.05	3.25	3.90
0.10	5.51	6.68
0.15	6.68	7.84
0.20	7.95	8.96
0.25	8.69	9.64
0.30	9.28	10.64

200 cc. 2% gelatin solution at room temperature and the resulting ratio of free amino- to total nitrogen determined at once, after 30 minutes, and after one hour. The amounts of ficin in the 25 cc. samples were varied from zero to 0.30 gram but the amounts present in the 25 cc. aliquots of the feces solution never exceeded the equivalent of 0.05 gram. A straight-line relationship was assumed to hold sufficiently well between 0 and 0.05 gram to permit interpolation. The original ratio in the gelatin was 2.92%. The results of the standardization are recorded in table 1, the *absolute* rise in the ratio for 30 and 60 minutes over the ratio as determined at once being recorded in relation to the amounts of ficin contained in the 25 cc. sample of the same.

The enzyme content of the 24-hour samples of feces was determined by direct proportion; the data used were those given in table 1 for a hydrolysis period of one hour. The 25 cc. aliquot of the feces solution was mixed with 200 cc. 2% gelatin solution. This solution plus 25 cc. H₂O had, as stated above, a ratio of free amino- to total nitrogen of 2.9%. After one hour hydrolysis at room temperature the ratio was determined and corrected for the nitrogen introduced into the feces solution. From this ratio there was subtracted the original ratio determined on the gelatin (2.92%). To apply a further correction for the small enzymatic activity encountered in normal feces, an average was made of the values similarly obtained on several control periods when no ficin had been administered. This average amounted to 0.75% absolute increase under identical conditions (see table 2) for

nitrogen total nitrogen ratio
Furthermore, experiments of
of the dialysate proportional to

One of the remarkable properties of continued medicinal use, is that the ability obviously does not accord with that of proteolytic enzymes but a member of the pig *Ascaris* provide ample confirmation (3). The helminths are rapidly attacked by the extensive lesions of the body wall. The power of locomotion of the worms leaves some time. However, long-continued active death and almost complete disintegration of the worms in the nitrogen content of the solution in which they were kept.

Site of action. In an attempt to determine the site of action of the enzyme on the live *ascaris* is on the exterior body surface or through the body wall, the following experiment was carried out: 100 worms were divided into three groups of five worms each. Group I received 200 cc. 0.1 per cent ficin made in Locke's solution; Group II received the same volume of Locke's solution without the ficin. Group III received the same volume of Locke's solution but, whereas the worms of Group I in the ficin solution but, whereas the worms of Group II had their alimentary tract closed by ligation as described by Brown (4), the worms of Group III were left unligated. All three groups were incubated at 37°C. at 15 minute intervals up to 150 minutes. At the end of that period the worms of Groups I and III, while retaining the power of locomotion, had a badly blistered body surface whereas Group II (controls) showed no damage. However, no difference could be observed between Groups I and III; since ligation of the alimentary tract was without result, the action of the enzyme was purely on the exterior surface of the worms. Moreover, the increase in the nitrogen content of the solution as digestion proceeded was practically identical in Groups I and III in spite of the inability of the enzyme to gain entrance to the intestinal tract of the worms of Group I. A similar experiment in physiological saline solution gave the same results.

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ment with a sample of papain paste² produced similar results, the recoveries having reached a maximum of about 63 per cent of that administered.

It is, of course, recognized that such a heavy dose of a highly irritating enzyme (cf. 6) produces abnormal conditions in the intestine. In the above-described experiments with ficin the resulting feces were patently abnormal, being soft and quite red in color, undoubtedly indicating some bleeding. However the animals quickly recovered their normal condition.

CONCLUSIONS

Further investigation of the properties of the fig enzyme (ficin) have shown that:

1. The action on *Ascaris lumbricoides* is on the exterior of the live worm and not through its alimentary tract.

2. The similar effect on *Trichuris* in the cecum is explained by its ability to pass through the digestive tract of the dog and to be excreted in the feces with a large share of its proteolytic activity intact.

We wish to acknowledge the assistance of The Samuel S. Fels Fund for the means to carry on this investigation.

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² Kindly furnished by Dr. Arnold K. Balls of the U. S. Department of Agriculture, Washington, D. C.

INTRAVENOUS INJECTIONS OF SOLUBLE BISMUTH COMPOUNDS: THEIR TOXICITY, AND THEIR SOJOURN IN THE BLOOD AND ORGANS¹

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Intravenous injection of water soluble bismuth compounds is not used clinically because of the relatively high toxicity, which had been shown experimentally and confirmed in the few clinical trials that have been reported.

H. Beerman, 1932 (1), found reports of five *acute deaths of human patients* from intravenous injection of bismuth compounds. The following abstract is typical of such occurrences: S. H. Curtis, 1930 (2), had used intravenous injections of a solution of "bismuth tartrate" (presumably sodium bismuth tartrate) in ten patients, without any reactions. He injected the equivalent of 15 mgm. of bismuth (approximately 0.25 mgm. per kilogram of body weight) in 5 cc. of sterile distilled water, weekly for ten doses. An eleventh patient, however, a young man in good physical condition, reacted to each of the ten injections with abdominal pain and one or two watery stools. A month later another course was started, but the patient died within five minutes of the first injection, with stormy symptoms of colloidoclastic shock, cyanosis, collapse and immediate loss of consciousness.

The introduction of the slow intravenous drip method of administering relatively large doses of neoarsphenamine and mapharsen (3), suggested to Dr. H. N. Cole that similarly slow injection of bismuth salts might decrease their toxicity sufficiently so that they could be utilized, especially as the experimental fatalities from intravenous bismuth administration often occur suddenly during or very shortly after the injection, suggesting flocculation, which could perhaps be avoided by slow intravenous injection. In any case the phenomena of intravenous bismuth injection called for further study, especially in comparison with the central nervous symptoms that are so prominent in poisoning by alkyl bismuth (4), and which are absent in ordinary bismuth poisoning. The difference could conceivably be due to the more rapid absorption of the alkyl compounds. It also appeared desirable to make quantitative studies of organ distribution and of blood concentration of bismuth when the dosage entering the blood could be absolutely controlled. For comparison, studies were made with single injections in ten to thirty minutes ("fast injection"), and in four to ten hours ("slow

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injection"), and with injections, slow or fast, repeated daily up to five days. The studies were begun on unanesthetized rabbits, and extended to dogs, without and with anesthesia.

Method for prolonged intravenous injection of unanesthetized rabbits.—The animal is placed in a rabbit injection box like that described by D. E. Jackson, but with several large holes bored through the sides and bottom for ventilation. The head protruded through the V-shaped cut and was freely movable. The box was placed in a trough tilted to drain urine through the bottom holes. After the hypodermic needle (usually gauge 22 or 24) had been inserted into the marginal ear vein and clamped to the ear by means of an artery clamp, the ear was fastened to the lid. This arrangement served at least twelve hours, if the needle was nearly parallel to the vein, and the flow of solution was not too slow. The solutions for slow injections were prepared by dissolving the calculated daily amount of the bismuth compound together with 12.5 grams of dextrose in 250 cc. of distilled water. The mixture was placed in a liter flask which was painted with aluminum on the outside to retard the action of light. A Murphy drip tube, previously calibrated for size of drops, was then attached and the flow regulated by means of a pinch clamp. Under ordinary conditions the flow was 0.5 cc. (about eight drops) per minute, the total injection lasting about eight hours. Three types of water soluble bismuth compounds were used, as represented by *sodium bismuth citrate*, *sodium bismuth thioglycollate* (thiobismol) and *sobisminol*.³ The dilute solutions of these compounds flocculate slowly on standing, first the thioglycollate, then the citrate, and last the sobisminol. Under the conditions of our experiments a Tyndall effect was observable in one to four, six to eight and eight to twelve hours, visible flocculation in four to six, eight to sixteen, and sixteen hours, respectively. None of the fresh solutions produced visible turbidity in dog or rabbit serum in less than two hours when added *in vitro*.

TOXIC EFFECTS IN RABBITS. The outcome of the injections may be grouped as acute fatality, delayed death, and indefinite survival. The *acute deaths* include the animals that died during or shortly after an injection. The symptoms were typically those of flocculation shock. Death was preceded by dyspnea, salivation and asphyxial convulsions. Autopsy showed pulmonary emphysema ("butterfly lung"). The *delayed deaths* occurred in 12 hours to 27 days, the time generally varying inversely with the dose. Death was due to nephritis. Extensive edema was present in the animals. No other signs were observed, except that the characteristic black deposits of bismuth occurred in the cecum. The *indefinite survivals* were killed 30 to 57 days after injection. The animals in this group did not show any signs of toxicity during life, and nephritic changes when present were of a much less degree than in the delayed deaths.

The nephritis and edema are evidently related to the severity of the intoxication and the time of survival. Edema was observable in about 20 per cent of the acute, 30 per cent of the subacute, 65 per cent of the delayed, 10 per cent of the late fatalities, and in none of the animals that survived indefinitely. Severe nephritis was present in all the fatalities. It was much more moderate in the

³ *Sobisminol Solution, N.N.R.*, contains "a complex organic bismuth product the chemical nature of which has not been fully established. It is obtained by dissolving the products of the interaction of sodium bismuthate, triisopropanolamine and propylene glycol in a mixture of propylene glycol and water. Each cubic centimeter of the solution contains between 19.5 and 20.5 mgm. of bismuth and 0.5 cc. of propylene glycol."

animals that survived and were sacrificed after four weeks; in some of these no nephritis was noticeable on gross examination.

Fatal doses.—Table 1 presents the fate of the rabbits with *single fast* injections of the various bismuth compounds, arranged in descending order of dosage. They show that the LD_{66} (the dosage that is lethal for two thirds of the animals), when injected intravenously into rabbits in about ten minutes, corresponds approximately to 4 mgm. of Bi per kilogram of body weight with sobisminol, to 2.5 mgm. with the citrate, and to 3.5 mgm. with the thioglycollate. Excluding the acute fatalities from these data does not change the LD_{66} . The difference in the LD_{66} of these compounds, as related to fast intravenous injection, is not large and may fall within the limit of statistical error. Our figure for sobisminol agrees closely with those given by Hanzlik, Lehman and Richardson (5), viz. 4.2 for LD_{66} , 6.3 for LD_{100} ; that for sodium bismuth citrate approximates that of Longley, Clausen and Tatum (6). With thioglycollate, Grubitz, Lyons and Perkins (7), reported an LD_{100} of 8 to 10 mgm., which is almost three times our LD_{66} figure.

Table 2 presents the toxicity of "*slow injections.*" With these, the LD_{66} is 10.5 mgm. with sobisminol, 5.0 with the citrate, 2.8 with the thioglycollate. Comparison of the fast and slow injections shows that with the latter method the toxicity of all is reduced approximately by one half, excepting the thioglycollate, which appears actually more toxic with slow than with fast injections. This is due to the high incidence of acute fatalities during or at the end of the injection, and is perhaps caused by a physical change of the labile thioglycollate during the slow injection, or during the prolonged sojourn in the blood-stream which was shown by studies of its blood concentration in dogs; these data will be presented later in the paper.

Table 3 shows the fate of the rabbits with *repeated slow injections*, the aim being to administer five equal fractions on successive days, by the technic described for single slow injections. The results appear too irregular to assign a definite L.D. The complication is due chiefly to the incidence of fatalities during or shortly after relatively small final doses. Our impression is that spreading the dosage over five days decreases the *late* toxicity somewhat, but not to a practically important extent, and that it increases the liability to acutely fatal reactions in a very dangerous degree.

Incidence of acute deaths.—Animals that die during or within a few minutes after the injection show pulmonary distention and other phenomena of flocculation shock, differing materially from the fatalities that occur later. They evidently belong to a different category. In a sense they are "accidental," involving some peculiar factor which is not clearly related to the dosage. The fatalities with single fast injections occur with relatively small doses (3.5 and 2.5 mgm. for sobisminol, 5 mgm. for the citrate), and at a considerably higher dosage with slow single injections (14.2 and 38 mgm. for sobisminol; 15 mgm. for citrate; 34, 25, 18, 4 and 2.8 for thioglycollate). The incidence (the ratio of acute deaths to all injections) is about twice as great for fast as for slow single injections with sobisminol (fast, 2:9; slow, 2:20) and citrate (fast 1:9; slow

1:16). Thioglycollate shows a peculiar behavior, since none of the six rabbits with fast injections died, but 5 out of 9 did with slow injection. We suspect that this may be due to the instability of the thioglycollate solution during the

TABLE 1

Outcome of single fast intravenous injection of bismuth compounds for rabbits

The bismuth compound was dissolved in 5 cc. of 5 per cent dextrose and injected, generally at the rate of 0.5 cc. per minute, so that the total injection usually required 10 minutes.

DOSE AS MGM. BI PER KG.	SOBIRMINOL	Na Bi CITRATE	Na Bi THIO- GLYCOL- LATE
9			+ 1 d.
7			+ 4 d.
5	+ 4 d.	+ 5 m.	
5	+ 5 d.	+ 5 d.	
3.5	+ end of inj.		+ 21 d.
3.5	K 34 d.		+ 2 d.
3.5	K 37 d.		
3.0		+ 6 d.	+ 9 d.
3.0		+ 8 d.	K 37 d.
3.0		+ 10 d.	K 41 d.
3.0		K 41 d.	
2.5	+ during inj.	+ 11 d.	
2.5	K 35 d.	+ 14 d.	
2.5	K 48 d.	+ 33 d.	
2.0	K 35 d.		
LD ₅₀ (approximate)	4 mgm.	2.5 mgm.	3.5 mgm.

Abbreviations: d = day; m. = min; + = time of death; K = time of sacrifice; h = hour.

TABLE 2

Outcome of single slow intravenous injections for rabbits

Most of the injection volumes were 250 cc. in 8 hours; some 500-600 cc. in 10 to 14 hours.

DOSE AS MGM. BI PER KG.	SOBIRMINOL	Na Bi CITRATE	Na Bi THIO- GLYCOLATE
34-40	+ dur. inj. + 1 d.		+ dur. inj.
25-28	+ 3 d. + 5 d.		+ dur. inj.
18-20	+ 3 d. + 3 d. + 5 d. + dur. inj.	+ end of inj.	+ end of inj.
12-15	+ 6 d. + 8 d. + 8 d. + 19 d.	+ 1 d. + 3 d.	+ 4 d.
10-11	+ 5 d. + 7 d. + 10 d. + 10 d. K 38 d. K 39 d. K 58 d. K 70 d.	+ 8 d. + 9 d.	
9.5		+ 9 d.	
9.0		K 50 d.	
8.0		+ 4 d. + 4 d. + 2 d. + 3 d. + 10 d. + 9 d. + 18 d. K 57 d.	
7.0			+ end of inj.
6.0			+ dur. inj.
5.0			+ 14 d.
4.0			K 51 d.
2.8			K 51 d.
2.5		K 57 d.	
LD ₅₀ (approximate)	10.5 mgm.	5.0 mgm.	2.8 mgm.

long injection, although special care was taken to minimize this by protecting the solution against light and by renewing it at one to four hour intervals.

The incidence of acute deaths appears distinctly higher for repeated injections than for single injections, double in the case of sobirminol (3:14 repeated as

against 2:20 for single); four times for citrate (2:8 repeated, 1:16 single). For thioglycollate, however, the incidence of acute deaths was lower for repeated injections (1:4) than for single slow injections (5:9). The dosage at which acute death occurred is strikingly different for repeated and single slow injections. The amount of bismuth which was injected during the last of the series of repeated injections in which acute death occurred ranged between 0.7 to 2.5 mgm., median 1.6 mgm., while for single slow injections it ranged between 2.8 and 40 mgm., median 16 mgm. The median dose at which acute death occurred in

TABLE 3
Outcome of repeated (daily) slow intravenous injections for rabbits

MG. BI KGM. AT EACH INJ.	SORBIMINOL			Na Bi CITRATE			Na Bi THIOLYCOLLATE		
	Number of doses	Total Bi		Doses	Total Bi		Doses	Total Bi	
16.7	16.7; 7.3	24	+ 2 d.						
12	12; 12	24	+ 2 d.						
3.8							3.8; 3.8; 3.8; 1.9	13.3	+ end inj.
2.5	4½	11.5	+ during						
	4½	10.8	K 38 d.						
	3½	8.75	+ 24 d.						
	3	7.5	+ end inj.						
	1½	4.25	+ 15 d.						
2.0	5	10	K 39 d.	5	10	+ end inj.			
	5	10	K 39 d.	5	10	K 42 d.			
	5	10	K 39 d.	5	10	K 42 d.			
1	2½	2.7	+ during	5	5	+ end inj.	5	5	+ 8 d.
1	5	5	K 31 d.	5	5	K 28 d.	5	5	K 34 d.
1	5	5	K 43 d.	5	5	K 41 d.	5	5	K 48 d.
1	5	5	K 48 d.						
0.5				5	2.5	+ 27 d.			
Fast				5	2.5	K 33 d.			
LD ₅₀ —total bis- muth—mgm. per kgm. (ap- proximate)		8			7.5				

repeated injections is therefore only one tenth that for single slow injections. The proportion of such fatalities increased with the number of repeated injections, for of the twenty six rabbits injected repeatedly, none died acutely with the first or second injection, two died with the third, one with the fourth and three with the fifth injection. We are inclined to attribute the acute deaths from small doses after repeated injections to thrombosis and embolism, arising from trauma and inflammation of the delicate vein of the rabbit's ear. Flocculation between blood and the bismuth solution is not materially hastened by repeated injections, as was seen by layering the bismuth solutions over serum and also by mixing serum with the solution to obtain a bismuth concentration com-

parable to that during injection of intact animals. Flocculation was visible in $1\frac{1}{2}$ –2 hours and precipitation in 14–18 hours, alike in the serum of rabbits that had not been injected with bismuth, and of those that were killed 4–16 hours after receiving five slow intravenous injections of the various bismuth solutions.

Time of death in relation to dosage.—Figure 1 shows that the dosage has a marked effect on the time of survival, both with slow and with fast injections. The average dosage of bismuth that kills rabbits in one day is 18 mgm. per kilogram, with slow injection; half of this dosage requires an average of nine days to kill. With fast injection, an average of 9 mgm. is fatal in one day and half of this kills in five days. The curves are somewhat parabolic; they are similar for the three compounds and for fast and slow injections; the few data

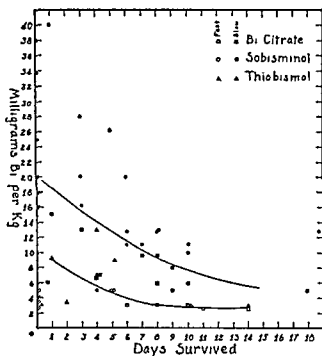


FIG. 1. RELATION OF DOSAGE TO SURVIVAL TIME, WITH INTRAVENOUS INJECTIONS OF BISMUTH SALTS

on repeated injections also run in the same direction. Rabbits that survive ten days generally recover.

Comparison of bismuth toxicity with intramuscular injections of watery solutions.—Even the slow intravenous injections are considerably more lethal to rabbits than intramuscular injections, for according to the data recorded in the literature the intravenous fatal dose is probably of the order of one fifth to one tenth that of the intramuscular injections.

More accurate comparisons are not feasible since the published data involve different criteria of survival and different periods of observation. For sobisminol, whose median LD_{50} we found to be 10.5 mgm. per kilogram by slow intravenous injection, Hanzlik, Lehman and Richardson (5), found a LD_{100} of 77 mgm. per kilogram for intramuscular injection, a ratio 1:7. For sodium bismuth citrate we found 5 mgm. for slow intravenous injection. Longley, Clausen and Tatum (6) give 10 mgm. as a "tolerated" intramuscular dose, a ratio

of 1:2. Leonard (8) reports 200 to 300 mgm. as the LD_{100} , a ratio of 1:40. For sodium bismuth thioglycollate we found 2.8 mgm. for slow intravenous injection; Grubitz, Lyons and Perkins (7) give the LD_{100} as 10 to 12 mgm., a ratio of 1:4.

TOXIC EFFECTS IN DOGS. *Intravenous toxicity for unanesthetized dogs.*—The animals were trained daily for a week by loose strapping with elastic bandage to an animal board, and by fixed extension of the leg in which injections were to be made. Following the training period there was no serious difficulty in injecting the bismuth in 500 cc. of 5 per cent dextrose solution into the pedalic vein over periods of $4\frac{1}{2}$ to 8 hours. Sobisminol was administered to all animals excepting one, which received the citrate. Three dogs that received a *single slow intravenous* injection of sobisminol in doses corresponding to 11, 7, and 7 mgm. bismuth per kilogram died in 3, 5 and 5 days respectively. Two injected with 5 mgm. of Bi as sobisminol and one with 3 mgm. as citrate survived indefinitely and were killed in 90, 36 and 60 days respectively. The LD_{50} lay between 7 and 5 mgm. per kilogram, i.e., about half the LD_{50} of rabbits given slow intravenous injections (10.5 mgm.). Dogs appear therefore somewhat more susceptible to intravenous bismuth poisoning than are rabbits, in agreement with the observations of Levaditi (9) and Brabant (10), but the difference is not beyond the range of statistical error. *Repeated slow injections* were made in two unanesthetized dogs each receiving five injections of sobisminol on successive days; one dog was given 2 mgm., the other 1 mgm. of Bi in each injection, making the total dose 10 and 5 mgm. respectively. Both survived until they were killed, after 38 and 34 days respectively. Although the data are few they indicate that perhaps a somewhat higher total dosage may be tolerated if it is spread over several days. The *symptoms* in delayed poisoning in dogs as in rabbits appeared to depend solely on the degree of kidney damage, and this in turn on the dosage of bismuth. Fatal cases developed the lethargy of uremia. If the dose was less than fatal, the animals remained in apparent good health during the observation period. When they were sacrificed the autopsy revealed varying degrees of kidney damage. There were no indications of bloody diarrhea, peripheral neuritis or encephalopathy.

Toxicity of intravenous injections in anesthetized dogs.—This was observed incidental to the study of the rate of disappearance of bismuth from the blood. The dogs were under barbiturate anesthesia (sodium isopropyl ethyl barbiturate 75 mgm. kgm. or sodium isopropyl allyl barbiturate 50 mgm. kgm.). The slow injections were made as in the unanesthetized dogs. Death occurred acutely at the end of the injection in the three dogs that received sobisminol in ten minutes; the dose of Bi was 2 mgm. per kilogram in one dog, 4 mgm. per kilogram in the others. The other 12 dogs survived at least $4\frac{1}{2}$ hours after the end of the injection of 4 mgm. per kilogram of bismuth; two of these dogs were injected with sodium bismuth thioglycollate in ten minutes, two with sobisminol in twenty minutes, two with sobisminol and six with thioglycollate for $4\frac{1}{2}$ hours. One of the latter died $4\frac{1}{2}$ hours and one 36 hours after the end of the injection. It would appear that sobisminol is especially liable to produce acute death in anesthetized dogs if it is injected rapidly, i.e., within ten minutes, and that sub-

acute death may occur even with slow injection of the thioglycollate in the dose of 4 mgm. of Bi per kilogram which did not cause death in any of the nonanesthetized dogs. The explanation of the apparent potentiating effect of anesthesia on the acute and subacute bismuth toxicity is not clear.

Sojourn of bismuth in the blood.—The difference in therapeutic and toxic effects of slow and fast intravenous injections of bismuth compounds would depend chiefly on the level of concentration that is attained and maintained in the

TABLE 4

Bismuth concentration in blood of dogs (mgm. Bi per 100 cc. of whole blood) after intravenous injection of soluble bismuth salts, in the ratio of 4 mgm. Bi per kgm. of body weight.

Barbiturate anesthesia

A. FAST INJECTIONS (THIOBISMOL IN 10 MIN.; SOBISMINOL IN 20 MIN.)								24 HOURS	PER CENT OF BISMUTH WHICH HAS LEFT BLOOD STREAM BY END OF INJECTION
Dog number	Body weight	Compound	Time (minutes) after end of injection:						
			0	10	30	90	270		
	kgm.								
2	17.0	Thiob.	0.482	0.282		0.111	0.105		89
6	17.7	Thiob.	0.355	0.214	0.220	0.177	0.094		91
11	24.7	Sobis.	1.16	0.64	0.355	0.200	0.120		73
12	15.5	Sobis.	0.800	0.308	0.208	0.132	0.036		82
B. SLOW INJECTIONS (4½ TO 5½ HOURS)									
			During injection			After injection			
			30 min.	90 min.	End	30 min.	90 min.	270 min.	
3	24.0	Sobis.	0.071	0.174	0.247	0.100	0.082		94
8	13.6	Sobis.	0.054	0.084	0.100	0.070	0.044	0.021	97
4	28.0	Thiob.		0.071	0.350	0.357	0.364	0.342	92
7	13.9	Thiob.	0.121	0.142	0.200	0.166	0.200	0.214	95
13	15.3	Thiob.	0.077	0.110	0.130	0.092	0.077	0.074	97
14	17.5	Thiob.	0.036	0.092	0.133	0.100	0.075	0.056	97
9	24.6	Thiob.							
		Plasma	0.069	0.383	0.440	0.114	0.300	0.182	87
		Cells	0.011	0.063	0.089	0.098	0.068	0.025	
10	24.6	Thiob.							
		Plasma	0.081	0.355	0.325	0.250	0.285	0.120	0.051
		Cells	0.040	0.032	0.076	0.098	0.068	0.051	0.051

blood. This was studied on dogs under barbiturate anesthesia (same as above), by injecting 4 mgm. of bismuth per kilogram of body weight, either as sobisminol or as sodium bismuth thioglycollate, as fast injections in 10 minutes for thioglycollate, in 20 minutes for sobisminol; and as slow injections in 4½ to 5½ hours for both. Samples of blood representing 5 cc. per kilogram were withdrawn at the end of the injection, and at 10, 30, 90 and 270 minutes after the end of the injection; in slow injections samples were also taken at a half hour and 1½ hours during injection. The samples, about 100 cc. each, were analyzed by the method described below for rabbit organs. The results are shown in table 4 as milligrams

Retention of Bi. in the Dog's Blood (median values)

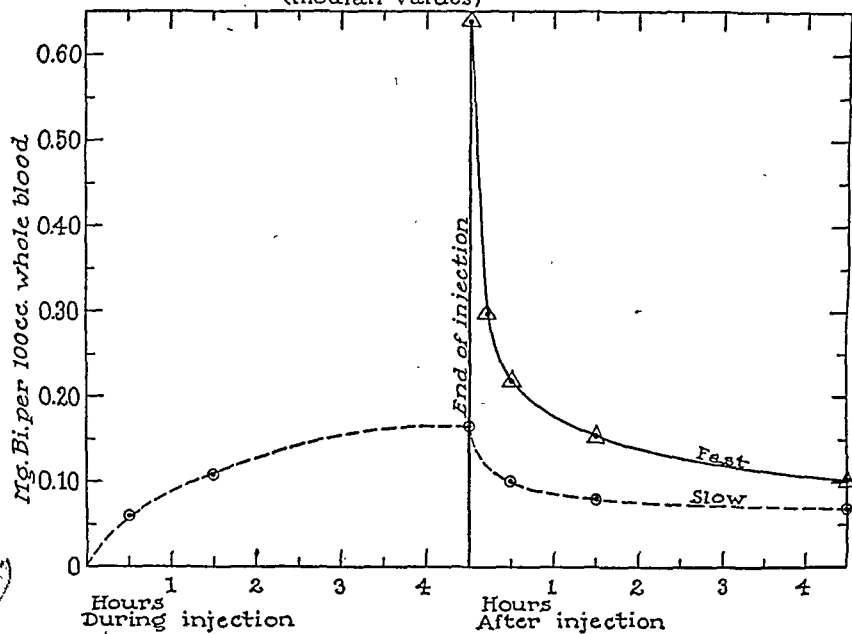


FIG. 2

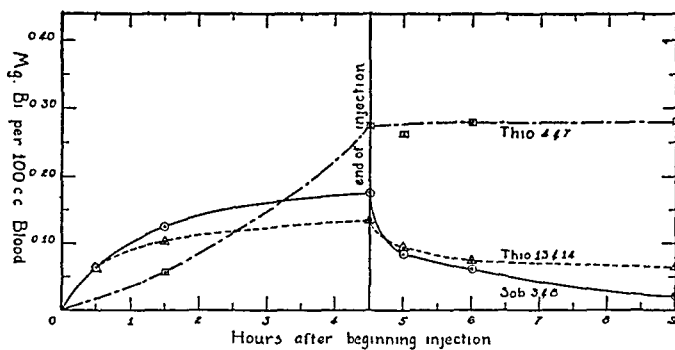
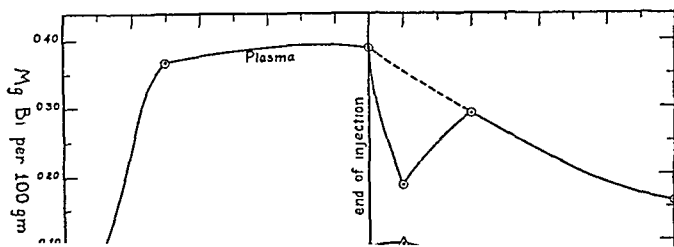


FIG. 3



of bismuth per 100 cc. of whole blood; the medians are plotted as curves in figures 2, 3 and 4.

With the *fast injections*, the fall of bismuth concentration is represented by parabolic curves which are fairly parallel in the four experiments, although the initial concentration was about twice as high for sobisminol injected in 20 minutes as for the thioglycollate injected in 10 minutes. The median concentrations, as shown in figure 2, averaged 0.641 mgm. Bi per 100 cc. at the end of the injection; this had fallen to somewhat less than half (0.295 mgm.) in ten minutes, to about one third (0.220 mgm.) in 30 minutes, to one fourth (0.155 mgm.) in 90 minutes; and to one sixth (0.100) in $4\frac{1}{2}$ hours. By this time the concentration curve was almost horizontal. With the *slow injections* ($4\frac{1}{2}$ hours) the median concentration of the six experiments, as shown in figure 2, rose slowly during the injection, in the form of a parabolic curve, to 0.167 mg. Bi per 100 cc. of whole blood, and then descended as a parabolic curve, to three-fifths (100 mgm.) in a half hour, to half (80 mgm.) in $1\frac{1}{2}$ hours, and from there it was almost horizontal. Comparing the median data and curves of the fast and slow injections, it appears that the bismuth concentration at the end of the injection is about four times higher in the fast than in the slow injection, but that the difference is of rather short duration. In about two hours after beginning the injection the blood concentration is about the same for fast and slow injections. The total bismuth equivalent of the blood expressed by the area of the median curves (the product of concentration multiplied by time) is about 19 per cent greater for the fast than for the slow injections, and in this sense and to this degree the fast injections would appear to have greater efficiency (toxic or therapeutic) than the slow injections. However, this difference may not be statistically significant.

Further examination of the six slow injections shows that they fall into three groups, each with two experiments of almost identical concentration curves, shown in figure 3: The two sobisminol animals (3 and 8) and two of the thioglycollate animals (13 and 14) give very similar figures, with the typical parabolic rise of concentration during the injection and the inverse parabolic fall after the injection. The other two thioglycollate experiments (4 and 7), however, gave a materially different curve: The ascending limb of the curve during the injection did not flatten out but reached a median level twice as high as that of the other two thioglycollate animals, and when the injection was stopped the concentration did not fall but continued practically horizontal for the $4\frac{1}{2}$ hours of observation. In other words, the bismuth did not leave the circulation, as it did in the other animals, indicating that the thioglycollate bismuth is liable to pass into nondiffusible, presumably colloidal form in the course of the injections.

FIG. 2 shows the median concentration after fast (solid line) and slow (broken line) injections and their time-relations.

FIG. 3 shows the average bismuth concentrations after slow injection of sobisminol (solid line) and two specimens of thioglycollate (broken and dotted lines).

FIG. 4 shows concentration of the average bismuth blood plasma (solid line) and corpuscles (broken line).

This tendency varies in different specimens, those of injections 4 and 7 coming from one lot, those of 13 and 14 from another.

The concentration curves of plasma and corpuscles were determined separately in two dogs killed by thioglycollate injections (of the same lot as 13 and 14). The median curves (fig. 4) show that thioglycollate bismuth enters and leaves the red blood cells much more slowly than the plasma, so that the bismuth concentration ratio of plasma: corpuscles stands about 4.4:1 at the end of the injection, 4:1 at 4½ hours after the injection, and 1:1 at the end of 24 hours.

The speed with which the bismuth leaves the blood during the injection may be calculated by deducting from the quantity injected (4 mgm. per kilogram) that which remains in the blood at the end of the injection (the concentration, in milligram Bi per 100 cc. of blood, multiplied by the normal blood volume, 90 cc. per kilogram of body weight). The percentage of bismuth which has left the blood by the end of the injection is shown in the right-hand column of table 4. It averages 90 per cent for thiobismol injected in 10 minutes; 78 per cent for sobisminol in 20 minutes, 95 per cent for thiobismol in 5 hours, 95.5 per cent for sobisminol in 5 hours.

THE BISMUTH CONCENTRATION OF RABBIT ORGANS AFTER INTRAVENOUS INJECTION OF BISMUTH. This is important especially as a basis of comparison with clinical administration, for while it is questionable whether bismuth is therapeutically active after it has been deposited in the organs, their bismuth content reflects the amount of bismuth that has passed through the circulation. Analytical data on clinical material are available for such comparisons, as are also results with intramuscular administration to animals. The analyses of the present series were performed chiefly by G. H. Mangun, with the method used in previous work in this laboratory (11), with the following modification which shortened the time necessary for analysis. Wet ashing was replaced by muffle ashing at 500°C. for 12 to 24 hours, after about 1 to 100 grams of tissue had been evaporated with five times its weight of concentrated nitric acid and a few drops of caprylic alcohol to prevent foaming. The reading was made with a Klett-Summerson photoelectric colorimeter. An average determination can be completed in 24 to 48 hours.

The analyses give the bismuth content as milligrams of bismuth per 100 gram of organ with its natural moisture. Since it may be expected that the dosage would have a very material effect on the concentration, this has also been reduced to a unit dosage, giving a quotient:

$$\frac{\text{concentration} = \text{mgm. Bi per 100 grams of organ}}{\text{dosage} = \text{mgm. Bi administered per kilogram of animal}}$$

For convenience we may designate this as "bismuth retention quotient" (*B.R.Q.*). The time which elapsed between the injection of bismuth and the death of the animal is another factor which would influence the organ content. From this standpoint, the experiments were grouped under five headings: (A) Acute death, occurring within two hours after injection; (B) Early death, occurring within two hours to one day after injection; (C) Delayed death, occurring > one to

8 days after injection; (D) Late death, occurring > 9 to 24 days after injection; (E) Indefinite survival, killed in 30 to 69 days. A selection of 79 animals for analysis was made from these groups so as to give representation to fast and slow single injections and to repeated injections of various dose ranges of the three bismuth compounds studied. In practically all of these the bismuth was determined in the kidneys, liver, lung and spleen, and in a few animals also in other tissues; the data represent a total of about 350 bismuth determinations. The B.R.Q.'s were calculated and tabulated in relation to the three compounds studied, to their dosage, to the speed of injection, and to single and repeated injections, so as to learn what effect these factors have on the retention of bismuth. Space is not available for the display of the individual data, so that we must be content to summarize the results: When the bismuth-retention quotients are plotted in decreasing sequence against varying doses of the three compounds, these sequences are scattered so irregularly that the two factors, dose and compound, do not appear significantly to alter the quotients. Nor are there consistent and significant differences between fast and slow injections. Repeated injections give somewhat lower figures than single injections, presumably because part of the bismuth is eliminated between the first and last day of injection. The difference, however, is slight, except in one or two instances in which it may have been accidental.

It seems justifiable therefore to base conclusions on the medians of the entire series. These are shown (with the range) in table 5. The concentrations in the *kidneys* and *lungs* fall with time in fairly smooth parabolic curves. The *liver* concentration appears to rise for a day, but this may be accidental since the one-day data are few. It then declines parallel to the kidneys and lungs. According to the data of the smoothed curves, the concentration in kidney, liver and lung falls to about one half between the end of the first and fifth day after injection, to one fourth by the tenth day, to one eighth by the fortieth day. The *spleen* concentration follows a different course, rising markedly to the fifth day and then declining much more abruptly than that in other organs. It appears therefore that the kidneys and lungs store bismuth rapidly, the liver and spleen more slowly. Table 6 expresses the median concentration of Bi in these organs as percentages of the kidney concentration. The liver ratio is seen to rise progressively with the lapse of time, from 17.1 per cent immediately after injection to 46 per cent after 41 days, so that the liver retains bismuth more tenaciously than the kidneys. The ratios in the lung and spleen rise for 5 days, from 6.8 to 13.8 per cent for the lung, from 11 to 93 per cent for the spleen, but after this time they drop more rapidly than those in the kidney. The tenacious retention therefore appears peculiar to the liver. This may be connected with storage in the reticulo-endothelial system.

The bismuth concentration in the fluids is shown in condensed form in table 7. The number of animals is not sufficient to establish relations for dosage and bismuth compounds, except that these were less than the accidental variations. They are therefore disregarded. The principal deductions are, (1) that the bismuth concentration of the *urine and blood* sinks to insignificant traces after 6 or

7 days; the urine concentration averages about 10 times that of the blood, the peritoneal *edema fluid* about one and a half times that of the blood (presumably because of the lower concentration in the blood corpuscles). (2) Till the fourth day the blood concentration averages about one-fifteenth of the liver concentra-

TABLE 5
Bismuth concentration in organs of rabbits

	A. ACUTE DEATH (WITHIN 1 HOUR AFTER INJECTION)	B. EARLY DEATH (2 HOURS TO 1 DAY)	C. DELAYED DEATH (1 TO 8 DAYS)	D. LATE DEATH (9 TO 24 DAYS)	E. SACRIFICED (25 TO 60 DAYS)
Number of animals analyzed.....	12	6	23	13	25
Concentration of Bi (mgm. per 100 grams of fresh organ)					
<i>Kidneys</i>					
Range.....	1.94 -43.4	4.32 -8.62	0.57 -6.8	0.32 -2.7	0.05 -1.19
Median.....	8.0	6.12	1.78	0.83	0.27
<i>Liver</i>					
Range.....	0.14 - 8.6	0.52 -6.93	0.07 -3.58	0.06 -0.91	0.03 -0.28
Median.....	1.37	1.72	0.58	0.296	0.124
<i>Lung</i>					
Range.....	0.05 -11.9	0.07 -0.53	0 -1.09	0 -0.37	0 -0.05
Median.....	0.54	0.15	0.245	0.03	0
<i>Spleen</i>					
Range.....	0 - 5.9	trace-5.38	0 -6.05	0 -1.78	0 -0.235
Median.....	0.89	0.77	1.65	trace	0
Bismuth-retention quotient (concentration divided by dosage as mgm. Bi per kgm. of body weight)					
<i>Kidneys</i>					
Range.....	0.25 - 2.46	0.15 -1.72	0.077-1.36	0.036-0.360	0.005-0.180
Median.....	0.800	0.671	0.163	0.204	0.076
<i>Liver</i>					
Range.....	0.034- 0.890	0.082-0.176	0.009-0.266	0.018-0.091	0.003-0.087
Median.....	0.084	0.139	0.053	0.054	0.023
<i>Lung</i>					
Range.....	0.016- 0.313	0.013-0.029	0 -0.232	0 -0.074	0 -0.009
Median.....	0.061	0.022	0.020	0.002	0
<i>Spleen</i>					
Range.....	0 - 0.520	trace-0.359	0 -1.20	0 -1.78	0 -0.067
Median.....	0.101	0.083	0.210	trace	0

tion; the urine concentration averages about a third that of the kidney till the sixth day. After this the concentration falls much more rapidly in the fluids than in the organs. (3) The concentration in the gall bladder *bile* follows a somewhat different course: Only a trace is found at the end of the injections, but it remains at relatively high levels from one to 9 days (the latest that was examined). It averages two-thirds of the liver concentration and in one rabbit the bile

TABLE 6

Median bismuth concentration of rabbits, organs as percentage of kidney concentration

GROUP	MEDIAN TIME OF DEATH	LIVER	LUNG	SPLEEN
A	End of injection	17.1	6.8	11.1
B	1 day	28	2.4	11.0
C	5 days	32.7	13.8	93.0
D	10 days	36	3.6	trace
E	41 days	46	0	0

TABLE 7

Bismuth concentration in fluid of rabbits (at time of death)

TIME OF DEATH	NUMBER OF ANIMALS	BISMUTH CONCENTRATION, MG/M PER 100 CC. (RANGE) AND MEDIAN	BISMUTH CONCENTRATION AS PERCENTAGE OF CONCENTRATION IN OTHER FLUIDS OR ORGANS OF SAME ANIMALS (RANGE) AND MEDIAN
Urine in bladder at time of death			
End of injection (8 hours).....	4	(0.38-7.6) = 1.5	(4.6-127) = 27% of kidneys
1 day.....	4	(0.34-3.1) = 1.8	(4-51) = 29% of kidneys
3 to 6 days.....	6	(trace-0.89) = 0.64	(trace-70) = 33% of kidneys
7 to 9 days.....	7	(0-0.67) = 0.05	(0-11.2) = 3% of kidneys
19 days	1	trace	trace
28 to 57 days.....	4	0	0
Blood			
End of injection.....	1	0.065	3% of urine; 2.3% of liver
3 and 4 days	2	(0.06 and 0.08) = 0.07	(9.6 and 39) = 25% of urine; 7.3 and 9.2 = 8.3% of liver
7, 8 and 10 days.....	3	(trace to 0.195) = trace	trace
26, 56 and 69 days	3	(0 to 0.009) = 0	0
Peritoneal edema fluid			
3 to 10 days.....	4	(trace-0.167) = 0.045	4 days = 140% of blood; (13 and 20) = 16.5% of liver
Bile in gall bladder (sobisminol injected)			
End of injection	1	trace	
1 day	2	(1.9 and 3.03) = 2.5	(43 and 66) = 55% of liver
3, 6 and 9 days	3	(0.33 to 3.22) = 1.34	(44 to 230) = 85% of liver

concentration was $2\frac{1}{2}$ times that of the liver. In a rabbit dying on the first day after injection the bile and urine concentration were about equal: in another dying seven days after injection, the bismuth concentration in bile was ten times that in urine. Sobisminol had been injected in all rabbits whose bile was examined.

Bismuth determinations in other solid organs.—A rabbit that died acutely at the end of the slow injection of a large dose of sobisminol (38 mgm. Bi per kilogram) gave the following bismuth concentrations (mgm. Bi per 100 grams): Skeletal muscle, 0.31; bone, 0.333; brain, 0.42; heart muscle, 2.8; intestines, 2.8. The concentration in the heart muscle and small intestines was about nine-tenths that of the liver; in the skeletal muscle and bone, about one tenth. The relatively high Bi content of the heart muscle may have been due to retention of bismuth flocculations in the coronary capillaries. The intestines offer a special problem by the precipitation of bismuth sulfide in the mucosa of the large intestines, especially about the spiral valve of the cecum. The small intestines usually contain only a fraction of the liver concentration of bismuth (0.071 mgm. per 100 grams in one day, 0.74 in three days, trace in fifty seven days, one rabbit each). The stomach in a one day rabbit contained 1.71 mgm. per 100 grams. The large intestines, however, contained 13.1 and 13.4 mgm. per 100 grams, in a one day and a three day rabbit, 20 to 200 times as much as the small intestines, 3 to 4 times as much as the kidneys, 4 to 5 times as much as the liver. The mucosa alone is responsible for the high concentration: In a one day rabbit, the concentration in the mucosa of the cecal valve was 17.6 mgm. per 100 grams, that of the underlying intestinal muscle was 1.55 mgm. per 100 grams. Even 57 days after injection the mucosa of the cecal valve of a rabbit gave 7.31 mgm. Bi per 100 grams, 53 times the concentration in the liver. The mucosal deposits are therefore retained with great tenacity, but it may be seen from the color that they undergo chemical changes; up to five weeks they have the brownish black color of bismuth sulfide, and subsequently they bleach slowly to the gray of metallic bismuth. Some of the animals sacrificed after 10 weeks showed no bismuth stain. The feces examined in one rabbit, which died three days after injection, had a bismuth concentration, 5.97 mgm. per 100 grams, 30 times the concentration in the urine.

Comparison with the data of other authors shows a bismuth retention quotient in rabbits's kidneys and liver identical with ours for the intravenous injections of sobisminol (Hanzlik, Lehman, and Richardson) (5). The *intramuscular injections of watery solutions* (sodium bismuth citrate and tartrate, Leonard *et al.*; sobisminol, Hanzlik *et al.*) give organ concentration of 6 to 14 per cent of equal doses by intravenous injection. With *intramuscular injections of oil suspensions* of the tartrate or citrate (Leonard *et al.*), the difference is not so great, but the organ concentration averages less than half (24 to 45 per cent) of that of equal doses by intravenous injection.

THE BISMUTH CONCENTRATIONS IN THE ORGANS OF DOGS. These were studied in nine animals, some with and some without anesthesia, with intravenous injection of 4 to 13 mgm. of Bi per kilogram, in the form of the three compounds,

2 with fast and 7 with slow injections. The data are irregularly distributed among these factors, so that these may be disregarded for this study. The summarized results are shown in table 8. Comparison of this with table 5 in which the results for rabbits are similarly arranged, show that the concentrations in all the organs tend to run several times higher for dogs than for rabbits with equal doses. The cause of this is not known, but it is conceivable that rabbits eliminate bismuth faster, by urine and by precipitation of sulfide in the intestinal mucosa, especially in the earlier periods. This may also explain why the intravenous toxicity of bismuth is somewhat lower with rabbits.

Total bismuth distribution in the body.—Table 4 showed that an average of 86

TABLE 8
Bismuth concentration in organs of dogs

	A AT END OF FAST (10 MIN) INJECTION	B 4½ HOURS AFTER END OF FAST (7 MIN.) IN- JECTION	B 4½ HOURS AFTER END OF SLOW (4½ HOUR) INJECTION	C 2 TO 4 DAYS AFTER SLOW INJECTIONS	E 34 TO 42 DAYS AFTER SLOW INJECTIONS
	Number analyzed				
	1	1	2	2	3
Concentration of Bi (mgm. per 100 grams of fresh organ-range and average)					
Kidney	5.40	13.1	8.71 - 26.0 = 17.36	6.00 - 5.30 = 5.65	0.51 - 1.44 = 1.25
Liver	0.92	0.21	0.13 - 0.14 = 0.14	0.58 - 0.71 = 0.65	2.05 - 0.54 = 0.53
Lung		0.19	0.57 - 0.15 = 0.36	0.063 = 0.063	0.026 - 0.054 = 0.050
Spleen	0.25	0.30	0.18 = 0.18	0.31 - 0.38 = 0.35	0.044 - 0.145 = 0.144
Blood	1.06	0.094	0.21 - 0.021 = 0.12	0.014 - 0.013 = 0.014	
Bismuth-retention quotient (concentration divided by dosage, mgm. per kgm. of body weight)					
Kidney	1.35	3.28	2.13 - 6.5 = 4.32	0.86 - 0.48 = 0.67	0.10 - 0.25 = 0.11
Liver	0.23	0.053	0.032 - 0.036 = 0.034	0.082 - 0.064 = 0.073	0.010 - 0.107 = 0.041
Lung		0.046	0.143 - 0.037 = 0.090	0.009 = 0.009	0.004 - 0.010 = 0.005
Spleen	0.064	0.075	0.045 = 0.045	0.044 - 0.034 = 0.039	0.009 - 0.029 = 0.011
Blood	0.265	0.023	0.053 - 0.005 = 0.028	0.002 - 0.001 = 0.002	

per cent of the administered bismuth has left the blood stream of dogs in the 10 to 20 minutes occupied by the fast intravenous injection and an average of 95 per cent has left during the slow intravenous injections lasting five hours. The major part of this has gone into the tissues, the remainder being excreted by the urine and feces. Table 9 presents the amounts found in the kidneys, liver, lungs, spleen and blood of dogs after various intervals up to 42 days; and table 10 gives the corresponding and more numerous data for the kidneys, liver and lungs of rabbits. These tables show the actual amounts in each organ, as milligrams of bismuth, and the percentage of the injected dose which this represents. The data for rabbits are shown as medians and range, to save space, without differentiation as to preparation or speed of injection, since it has been found that these do not materially alter the retention.

It will be seen that the percentage distribution of bismuth among kidney, liver and lung is of the same order of magnitude in the dogs and rabbits. During and within two hours after the injection about 3 to 5 per cent of the injected bismuth has gone into the kidneys, 6 to 10 per cent into the liver, 0.4 per cent into the lungs, 0.12 per cent into the spleen. During the remainder of the day the percentage rises somewhat in the kidneys (to 7-10 per cent), and falls somewhat in the liver (to 1-4 per cent) and also in the spleen (to 0.1 per cent). Within the next week the percentage in all the organs has fallen considerably, in the kidney

TABLE 9

Total bismuth in organs of dogs after intravenous injections (number of animals analyzed, as in table 8)

	A AT END OF 10 MIN. INJECTION	B 4½ HOURS AFTER END OF FAST INJECTION	D 4½ HOURS AFTER END OF SLOW INJECTION	C 2 TO 4 DAYS AFTER SLOW INJECTIONS	E 34 TO 42 DAYS AFTER SLOW INJECTIONS
Dose of Bi, mgm. per kgm. body weight.....	4	4	4	7 and 11	5, 5 and 13
Total Bi injected, mgm.....	88	70.8	54.4 and 55.6	40.0 and 73.2	24.1, 37.0 and 72.2

Total Bi (mgm.) in organs

Kidneys (both).....	2.4	6.70	6.56 - 5.14	1.35 - 0.90	0.085-0.405 = 0.182
Liver.....	8.46	1.00	0.378- 0.425	1.09 - 2.06	0.135-1.964 = 0.931
Lung.....		0.268	0.152- 0.80	0.026- —	0.022-0.044 = 0.037
Spleen.....	0.11	0.14	0.047- —	0.028-0.048	0.006-0.027 = 0.018
Blood.....	20.42	1.50	0.257- 2.677	0.09 - 0.09	

Percentage of injected Bi in organs

Kidneys (both).....	2.80	9.46	1.20 - 9.2	3.44 - 1.22	0.35 - 0.585 = 0.49
Liver.....	9.88	1.41	0.69 - 0.78	2.72 - 2.82	0.56 - 2.88 = 2.57
Lung.....		0.38	0.33 - 1.4	0.065- —	0.06 - 0.10 = 0.09
Spleen.....	0.12	0.19	0.086- —	0.07 - 0.065	0.025-0.05 = 0.038
Blood.....	23.1	2.12	0.47 - 4.8	0.22 - 0.12	

and liver to about 2.5 per cent, in the lungs to 0.1 per cent, and in the spleen to 0.067 per cent. The decrease continues and in 4 or 5 weeks the kidneys contain about 0.45 per cent, the liver relatively more, (about 1 per cent), the lungs and spleen each about 0.034 per cent. These averages are approximate combinations of the dog and rabbit data.

COMPARISON WITH CLINICAL ORGAN BISMUTH CONCENTRATION.—Analytical data on the autopsy organs of twenty two patients treated clinically by courses of intramuscular injections of bismuth preparations, generally subsalicylate, were published by Sollmann, Cole and Henderson (12). Table 11 shows the median concentration, regardless of dosage, for the principal organs of these patients, in comparison with the concentrations of our rabbits and dogs after single and frac-

tionated intravenous injections. At the end of the single intravenous injection in the animals the organ concentration averaged about twice as high as after a series of courses of intramuscular injections in the patients. The kidney concentration fell to about half the clinical level in a week; but the concentration in the other organs remained about the clinical level for two months. The intravenous dosage was higher than in a single clinical injection, but the median of the total clinical dosage is about 15 mgm. Bi per kilogram of body weight, which is two or three times the median intravenous dosage.

The patients averaged a kidney content of 4.5 mgm. per 100 gram for each gram bismuth injected, equivalent in a 70 kgm. patient to a B.R.Q. of $4.5:15=$

TABLE 10

Total bismuth in organs of rabbits after intravenous injections

	GROUP A	GROUP B	GROUP C	GROUP D	GROUP E
	Time of death after injection				
	Within 2 hours	2 hours to 1 day	2 to 8 days	9 to 24 days	25 to 60 days
Number of animals included.....	8	3	10	12	23
Median dose of Bi(mgm. per kgm.).	11.5	6.0	7.0	5.5	5
Total Bi (mgm.) in organs, average and range					
Kidneys (both).....	2.04 (0.34-5.08)	1.239 (0.84-1.88)	0.513 (0.13-1.53)	0.220 (0.043-0.57)	0.072 (0.013-0.131)
Liver.....	2.41 (0.163-6.83)	0.683 (0.62-0.78)	0.452 (0.055-1.07)	0.231 (0.034-0.950)	0.118 (0.020-0.296)
Lungs.....	0.181 (0.015-0.71)	0.033 (0.019-0.049)	0.019 (0.041)	0.014 (0.078)	0.0005 (0-0.006)
Percentage of injected Bi in organs (average)					
Kidneys (both)....	4.95	7.03	2.60	1.208	0.448
Liver	5.75	3.72	2.23	1.535	0.839
Lungs	0.41	0.182	0.095	0.079	0.034

0.3. This quotient is less than that at the end of intravenous injection in rabbits (median 0.8), and even after one day (median 0.65). It is about equal to that of five days after intravenous injection (median 0.35). Beyond these times it rises above that of the intravenous quotient, which has the medians of 0.18 in 10 days, 0.1 in 20 days, 0.08 in 40 days. The intravenous injection therefore secures a higher initial concentration, but beyond five days after a single intravenous injection the concentration falls below that produced by the intramuscular courses.

From a smoothed curve of the data of table 5, one may calculate the amount of bismuth that must be given by a single intravenous injection to secure a concentration of 3.33 mgm. Bi per 100 gram of kidney, at any time after the injection: Immediately after injection, this approximates 4 mgm. per kilogram; for one day after injection, 5 mgm. per kilogram would need to be injected; to

have this concentration on the fifth day would require the initial injection of 10 mgm. per kilogram; for the tenth day, 20 mgm.; for the twentieth day, 33 mgm.; for the fortieth day, 40 mgm. Even the smallest of these quantities, 4 mgm. per kilogram of 280 mgm. for a 70 kgm. man would appear far too dangerous. Klaunder (3) injected patients twice a week intravenously with colloidal bismuth hydroxide, equivalent to 1 mgm. of bismuth per kilogram of body weight, without untoward effects, but the acute fatality of Curtis occurred with about 0.25 mgm. of bismuth (as sodium bismuth tartrate) per kilogram of body weight.

ANTISYPHILITIC EFFICIENCY OF INTRAVENOUS BISMUTH PREPARATIONS.—The question whether the extra high bismuth concentrations that are secured temporarily by intravenous injection add materially to the efficiency of bismuth

TABLE 11

Median concentration of bismuth (mgm. per 100 grams of fresh tissue), regardless of dosage

SERIES	KIDNEY	LIVER	LUNG	SPLEEN	BLOOD
Patients after clinical courses of intramuscular injections.....	3.33	0.68	0.085	0.155	0.05
Intravenous, rabbits					
End of injection.....	7.67	1.37	0.538	0.893	0.065
1 day to 1 week.....	1.69	0.520	0.250	1.40	0.07
9 to 24 days.....	0.831	0.164	0.038	trace	trace
28 to 60 days.....	0.268	0.182	0	0	0
Intravenous, dogs					
End of injection.....	15.4	0.92		0.25	1.06
4 hours after injection.....	13.1	0.140	0.185	0.300	0.094
2 and 4 days.....	5.7	0.642	0.063	0.347	0.014
34 to 42 days.....	1.250	0.533	0.050	0.144	

treatment of syphilitic infections can only be answered by direct experiments. Dr. Hanzlik has started to investigate this on rabbits.

SUMMARY AND CONCLUSIONS

Three special water-soluble bismuth compounds—sodium bismuth citrate, sodium bismuth thioglycollate, and sobisminol, were injected intravenously into rabbits and dogs, comparing the "drip method" of very slow injection of dilute solutions extending continuously over four to ten hours, with injection of the same doses at the usual rate of ten to thirty minutes; the entire amount being introduced either on one day, or spread over several successive days, up to five. The toxicity of the citrate and of sobisminol with the very slow injections was about half that with the ordinary speed; this does not apply to the thioglycollate solution (presumably because this is more unstable).

Spreading the total dosage over several successive days decreases somewhat

the nephritic toxicity but increases the liability to acutely fatal reactions in rabbits, probably by thrombosis from traumatic vascular inflammation.

Acute fatalities during or within a few minutes after the injection occur also with single injections, about twice as frequently with fast injections (about 20 per cent of all fatalities) than with slow (about 10 per cent); but they are 2 to 4 times more frequent with repeated injections. They have little or no relation to the dosage and present the phenomena of colloidoclastic shock, while the ordinary bismuth phenomena are nephritic.

Death from bismuth nephritis occurs in several hours to 24 days after the injection, the time being generally inverse to the dose; for instance, half of the dosage that kills in one day is fatal after 9 days for slow, after 5 days for fast injections.

The fatal dose by intramuscular injection appears to be of the order of 5 to 10 times higher than that by slow intravenous injection, for rabbits.

The fatal dose of bismuth by intravenous injection averages somewhat smaller for dogs than for rabbits. The symptoms in dogs are uremic, without the bloody diarrhea, peripheral neuritis or encephalopathy that are conspicuous in poisoning by alkyl bismuth.

The sojourn of the injected bismuth and its concentration in the blood were studied on dogs. With the fast injections the concentration falls rapidly as a parabolic curve. With the slow injections it rises parabolically during the injection and falls similarly when the injection ceases. With fast injection the maximal concentration in the blood is about four times as high as that attained by slow injection, but by the end of two hours the concentration is about the same in both cases. The product of average concentration and total time is about one-fifth higher with fast than with slow injection of a given dosage.

The bismuth concentration of the organs varied directly with the dose and inversely with the time elapsed since the injection. The nature of the bismuth compound and the speed of injection are not significant. The concentrations in the kidneys (which are richest in bismuth) and in the lungs fall with time as fairly smooth parabolic curves. The concentration in the liver falls more slowly, so that its average ratio to that in the kidneys rises for 17 per cent on the first day, to 46 per cent at 41 days. The concentrations in the blood and urine sink to insignificant traces after 6 or 7 days; in the bile the Bi rise is slower and more persistent.

The bismuth concentration in the organs of dogs averages several times higher than for rabbits, with the same dosage and time. This suggests slower elimination of Bi by dogs (by the urine and by precipitation of sulfide in the intestinal mucosa) and this would explain the higher toxicity for dogs.

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THE FATE OF CERTAIN SYMPATHOMIMETIC AMINES IN THE BODY

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The fate of amphetamine in the body—that it is partially excreted as such (1, 2, 3), partially inactivated in the liver (4, 3) and deaminated by ascorbic acid (5) but not by amine oxidase (6, 7, 4)—seems well established. The purpose of the present investigation has been to determine whether a number of compounds related to amphetamine are excreted in the urine or totally inactivated in the body, and to examine these findings in the light of previous studies.

PROCEDURE. The amines studied in this work were

1. β phenyl-*n*-propylamine
2. γ phenyl-*n*-propylamine
3. α methyl- γ -phenylpropylamine
4. α phenyl-*n*-propylamine
5. β methyl- β -phenylisopropylamine
6. α isopropyl- β -phenylethylamine
7. α methyl- β -phenylisopropylamine

The method used to estimate the presence of these amines in urine was that first described by us (4) with some minor modifications (5). This has proved satisfactory for the present investigation. The only deviation from the method as previously described has been in the final determination. In this study we have used duplicate controls of 0.1 and 0.01 mgm. amine coupled with *p*-nitrobenzene-diazonium chloride and extracted with butanol. The galvanometric deflections for these controls have been plotted as the logarithmic function of the concentration on 2 cycle semi-log paper, a straight line drawn through the points, and the concentration of the sample read as the arithmetic coordinate where the galvanometric value intersected the curve. This has the advantages of doing away with a constant reference curve, as has been used in the past, and the corrections to such a curve when control determinations vary from it only slightly.

EXPERIMENTAL. *Amines excreted by the kidneys.* Ten milligram doses of compounds 3, 4, 5, 6, and 7 were given by mouth to 31 subjects who collected their total urine output for the first and second 24 hour periods following ingestion of the drug. The subjects were given the compounds in 20 cc. of water and directed to rinse the contents into a glass of water, drink that and follow it with half a glass of water. This was taken about half an hour before breakfast. Since most of the experiments were done during summer months, the urine volumes were often low. In every instance the compounds were excreted in considerable amounts, varying somewhat with the subject and the amine given.

Table 1 gives the results of excretion of 10 mgm. doses of α methyl- γ -phenyl-

propylamine (sulfate) by 10 subjects. Table 2 represents data on the excretion of 10 mgm. doses of α phenyl-*n*-propylamine, β methyl- β -phenylisopropylamine, α isopropyl- β -phenylethylamine and *o* methyl- β -phenylisopropylamine. Common to all of these is the fact that excretion of the amine is not related to the total urine volume within normal limits. The duration of excretion of determinable amounts of the amines was found to be about 48 hours, with one exception. Other trials have shown them to be present in the urine within 3 hours or less after the compound was swallowed.

However, there is some difference in the rate and extent to which these amines are excreted. From 20 to 54 (average 40.4) per cent of a given dose of α methyl- γ -phenylpropylamine was excreted in 24 hours. Figuring from averages, this represents about $\frac{2}{3}$ of the total amount (5.9 mgm.) excreted in 48 hours. In the case of α phenyl-*n*-propylamine 63 to 95 (average 78.9) per cent was excreted

TABLE 1

The excretion of 10 mgm. of α methyl- γ -phenylpropylamine taken orally by man

SUBJECT	1ST 24 HOUR PERIOD		2ND 24 HOUR PERIOD		TOTAL 48 HOUR PERIOD	
	Urine volume	Per cent excreted	Urine volume	Per cent excreted	Urine volume	Per cent excreted
R. D.....	1024	37.0	1500	26.4	2524	63.4
W. S.....	850	32.2	1250	22.0	2100	54.2
L. K.....	560	21.2	920	20.2	1480	41.4
O. E.....	625	36.6	655	11.8	1280	48.4
J. C.....	600	52.8	560	6.8	1160	59.6
W. R.....	860	47.5	975	30.1	1835	75.6
W. H.....	575	35.0	640	10.2	1215	45.2
B. B.....	1100	44.0	770	33.2	1870	77.2
A. P.....	980	44.1	550	22.4	1530	66.5
R. E.....	1098	54.2				
Average.....		40.4		20.4		59.1

within 24 hours. This comprises almost the total amount excreted; only an average of 2.8 of the total 81.7 per cent was excreted in the second 24 hour period.

The range of deviation from the mean was greatest for β methyl- β -phenylisopropylamine, being from 10 to 39.5 per cent excreted within the first 24 hours. The average of 24 per cent is $\frac{2}{3}$ of the total amount, 30 per cent, excreted during the experiment. The excretion of α isopropyl- β -phenylethylamine was the least of any of the compounds, being only about 20 per cent within 48 hours. In this instance, however, the duration of excretion of determinable amounts of the amine was 72 to 76 hours following ingestion. The total amount of *o* methyl- β -phenylisopropylamine excreted over 48 hours (88.2 per cent average) was but slightly greater than for α -phenyl-*n*-propylamine (81.7 per cent average) yet the rate of excretion of the former was much slower than the latter. In the case of *o* methyl- β -phenylisopropylamine about $\frac{2}{3}$ of the total amount (60.3 per cent) was excreted within 24 hours, whereas almost all (97 per cent) of the α phenyl-*n*-

propylamine was excreted during the first 24 hours. Acid hydrolysis of the urine did not increase the recovery of any of the amines.

TABLE 2

The excretion of certain sympathomimetic amines by man. 10 mgm. of each was administered in solution orally

SUBJECT	1st 24 HOUR PERIOD		2ND 24 HOUR PERIOD		TOTAL 48 HOURS	
	Urine volume	Per cent excreted	Urine volume	Per cent excreted	Urine volume	Per cent excreted
α phenyl-n-propylamine						
R. K.....	530	95.0	550	3.5	1080	98.5
S. B.....	1360	88.0	925	1.8	2285	89.8
I. E.....	415	73.7	690	3.6	1105	77.3
B. S.....	1210	63.4	1900	1.3	3110	64.7
H. G.	590	74.7	740	1.6	1330	76.3
Average.....		78.9		2.8		81.7
β methyl-β-phenylisopropylamine						
R. S.....	500	10.0	655	2.0	1155	12.0
E. K.....	1030	33.0	1720	13.2	2750	46.2
K. N.....	640	12.4	850	9.8	1490	22.2
C. L.....	375	10.0	610	13.4	985	23.4
M. O.....	750	39.5	520	12.5	1270	52.0
T. L.	710	39.4	760	8.1	1470	47.5
Average.....		24.0		6.0		30.0
α isopropyl-β-phenylethylamine						
M. A.....	1640	11.0	1640	5.5	3280	16.5
D. B.	700	9.3	550	12.8	1250	22.1
L. B.	830	14.1	900	10.8	1730	24.9
G. M.....	400	9.6	600	6.4	1000	16.0
M. S.....	765	10.3	578	9.2	1343	19.5
Average.....		10.9		8.9		19.8
o-methyl-β-phenylisopropylamine						
F. B.	1000	59.5	1190	36.1	2190	95.6
A. E....	420	56.0	340	24.5	760	80.5
F. B....	510	77.3				
R. L.	850	37.4	760	27.9	1610	65.3
B. T....	1335	71.6	1205	23.3	2541	94.9
Average.....		60.3		27.9		88.2

Using the Warburg respirometer and the technic described previously (8) we found that none of these five compounds was oxidatively deaminated in the presence of amine oxidase. This has also been found to be the case for amphetamine (6, 7, 4).

*Amines not excreted by the kidneys.*¹ Fifteen milligram doses of β -phenyl-*n*-propylamine and γ phenyl-*n*-propylamine were given orally to 8 subjects. Ten to 20 mgm. doses of each were injected subcutaneously daily for several days into 14 dogs. Neither of the compounds was excreted in the urine in more than an occasional trace by either the human subjects or the dogs. Thus the factor of destruction of the compounds before their absorption into the body was ruled out.

Since these 2 amines were not normally excreted following their administration, it was thought desirable to see if the dogs could be made to excrete these chemicals. To accomplish this 3 experiments were tried: 1) to inactivate the amine oxidase in the body, if possible, 2) by means of a substance (CCl_4) toxic to the liver and possibly other organs as well to impair their function, 3) to impair liver function alone.

Bernheim reported (9) that phenylhydrazine when added in concentration of 4.5×10^{-4} M to an amine oxidase preparation *in vitro* would totally inhibit the oxidative deamination of isoamylamine as substrate for the enzyme. In a study of the enzymic inactivation of substituted phenylpropylamines (8) one of us found that these two compounds were deaminated by amine oxidase. An attempt was made, then, to inject phenylhydrazine into dogs to inhibit if possible the amine oxidase and so cause β phenylpropylamine to be excreted.

*In this experiment 4 dogs were injected subcutaneously daily with 10 mgm. β phenyl-*n*-propylamine and their 24 hour urine specimens collected and analyzed for the amine.* Three days of control determinations were made to determine whether or not the compound was excreted. Total red and white blood cell counts, differential cell counts and hematocrit were determined on the controls and following the phenylhydrazine administration. Following the control periods the dogs were weighed and 100 mgm. of phenylhydrazine hydrochloride per kilo of body weight was injected subcutaneously in a volume of distilled water sufficient to dissolve it. The dogs usually vomited within an hour and appeared sick. The injections of the amine, collection of urine and blood counts were continued for from 4 to 6 days following the phenylhydrazine injections. During this time no β phenylpropylamine appeared in the urine though the dogs manifested severe phenylhydrazine poisoning. One of them, T, died 1 week following the injection of phenylhydrazine. Table 3 summarizes some of the findings in this experiment. In every instance, following the phenylhydrazine there resulted a severe hemolytic anemia, a rapid rise in the leukocyte count and a fall in the hematocrit readings. The differential cell counts showed many immature cells of both red and white series. Representative of the differentials is one day's count on dog C: Total w.b.c. 34,900, segmented neutrophils 20, nonsegmented neutrophils 58, meta myelocytes 6, myelocytes 4, promyelocytes 2, blasts 1, eosinophils 2, lymphocytes 7, erythroblasts 9 and proerythroblasts 3/100 w.b.c. There were numerous showers of platelets, polychromatophilia and anisocytosis. Urine from all the dogs was port red in color due to the destruction of erythrocytes and that from dogs S and T tended to form a loose

¹ Normally under the conditions of this experiment.

clot on standing in the refrigerator several days. It is doubtful whether we could have exceeded this dose of phenylhydrazine and have had any of the dogs live. It seems certain, then, that not enough of the drug could be given to animals to inactivate or even inhibit the amine oxidase to the point that β phenyl-*n*-propylamine was excreted by the animals.

Carbon tetrachloride is known to produce liver damage in dogs although its effect on the kidney is probably not so marked unless excessive amounts are given (10). An experiment similar to that for phenylhydrazine was performed, substituting in its stead CCl_4 and omitting the cell counts. Four dogs were used in this experiment and the condensed data are given in table 4. From the table it may be seen that in every instance CCl_4 in doses of 30 to 50 cc. orally caused β phenyl-*n*-propylamine to be excreted; this amounted to as much as 35 per cent of a given dose in one instance, dog D.

TABLE 3

*The result of an attempt to produce excretion of β phenyl-*n*-propylamine, 10 mgm. subcutaneously daily, by the subcutaneous administration of a single injection of phenylhydrazine hydrochloride*

DOG	AMOUNT OF PHENYLHYDRAZINE	ERYTHROCYTES		LEUKOCYTES		HEMATOCRIT		EXCRETION OF AMINE	
		Before	After	Before	After	Before	After	Before	After
	mgm. per kgm.	millions	millions	thousands	thousands	per cent	per cent		
C	75	7.40	1.60	9.15	37.20	50.6	10.3	0.00	0.00
E	100	7.38	1.76	9.50	26.65	49.5	27.5	0.00	trace
S	100	6.17	2.73	12.40	35.25	44.0	30.0	0.00	0.00
T*	100	4.48		10.6		44.0		0.00	trace

* Died 1 week after phenylhydrazine.

Before and after pertain to the injection of phenylhydrazine. The data given for after the injections are maximal determined values.

This effect of CCl_4 is probably a non-specific one, inhibition to the amine oxidase system occurring along with a general impairment of liver function. It was shown by Beyer and Skinner (4) that carbon tetrachloride administered to dogs caused them to excrete all of a given dose of benzedrine (amphetamine), and it has been amply confirmed that this compound is not inactivated by amine oxidase.

Wells (11) has shown that hydrazine, in doses of 50 mgm. per kilo subcutaneously, produces in dogs an almost specific central zone parenchymatous degeneration of the liver lobules without affecting other organs of the body. If CCl_4 could non-specifically inhibit amine oxidase along with the production of a parenchymatous degeneration of the liver and possibly other organs, it seemed possible that similarly hydrazine could be used to produce only a liver damage, thus in a measure evaluating the importance of the liver in the inactivation of these amines.

Three dogs each in two series were placed on daily subcutaneous injections

TABLE 4

The effect of carbon tetrachloride on the excretion of β phenyl-n-propylamine, 10 mgm. subcutaneously daily, by dogs

24 HOUR PERIOD	URINE VOLUME	MGM. EXCRETED	24 HOUR PERIOD	URINE VOLUME	MGM. EXCRETED
Dog D			Dog M		
2nd	625	0.00	3rd	300	0.00
3rd	590	0.00	4th	290	0.00
4th	30 cc. CCl ₄ orally		5th	50 cc. CCl ₄ orally	
6th	420	1.77	7th	145	0.29
8th	640	0.94	8th	355	1.63
12th	875	3.50	9th	195	trace
13th	570	2.52	10th	185	trace
Dog N			Dog O		
3rd	335	0.00	1st	755	0.00
4th	560	0.00	2nd	480	0.00
5th	50 cc. CCl ₄ orally		3rd	50 cc. CCl ₄ orally	
7th	415	0.42	4th	730	2.14
8th	440	0.41	5th	50 cc. CCl ₄ orally	
9th	655	trace	6th	385	0.65
10th	380	trace	7th	855	trace

TABLE 5

The effect of hydrazine on the excretion of β phenyl-n-propylamine and γ phenyl-n-propylamine by dogs

24 HOUR PERIOD	URINE VOLUME	MGM. AMINE EXCRETED	URINE VOLUME	MGM. AMINE EXCRETED	URINE VOLUME	MGM. AMINE EXCRETED
β phenyl-n-propylamine, 20 mgm. injected subcutaneously, daily						
	Dog P		Dog Q		Dog R	
2nd	390	0.00	215	0.00	765	trace
3rd	335	0.00	260	0.00	365	trace
4th	40 mgm. hydrazine sulfate per kgm. subcutaneously in 2 injections					
5th	655	0.32	355	0.65	1235	2.50
6th	150	0.57	335	0.92	225	2.35
7th	170	0.78	280	0.82	625	1.41
8th	125	0.25	380	1.14	650	0.94
γ phenyl-n-propylamine, 10 mgm. injected subcutaneously, daily						
	Dog U		Dog W		Dog X	
2nd	225	0.00	300	0.00	315	0.00
3rd	100	0.00	250	0.00	320	0.00
4th	40 mgm. hydrazine sulfate per kgm. subcutaneously in 2 injections					
6th	235	0.24	1050	0.42	265	0.26
7th	275	0.28	235	1.95	455	0.23

of 20 mgm. β phenyl-*n*-propylamine in one series, 10 mgm. of γ phenyl-*n*-propylamine in the other. Total 24 hour urine specimens were collected and tested for the respective amines. Neither series of dogs excreted the amines normally with the exception of dog R which excreted about $2 \mu/\text{cc.}$ of β phenyl-*n*-propylamine. After the control periods of 3 days the dogs were injected subcutaneously with 40 mgm. hydrazine sulfate per kilo in aqueous solution given in two divided doses at 6 hour intervals. It may be seen from table 5 that this dose of hydrazine caused the excretion of both β phenyl-*n*-propylamine and γ phenyl-*n*-propylamine by each dog of the respective series. It is not surprising that the excretion of the amines following hydrazine is no greater than that found. Bhagvat, Blaschko and Richter (12) have reported amine oxidase to be widely distributed in most of the organs of the body. If the distribution were equal throughout the organism, one would hardly expect an inhibition in or even elimination of that enzyme from one organ so to decrease the detoxication of these amines that they appear as such in the urine. Actually, it would seem that the liver is a principal site of inactivation of these amines whether or not it be entirely by amine oxidase.

INTERPRETATION. These results together with what has been found to be true for other similar sympathomimetic amines may be taken to establish certain fundamental concepts of the way an organism rids itself of these agents. The compounds α methyl- γ -phenylpropylamine, α phenyl-*n*-propylamine, β methyl- β -phenylisopropylamine, α isopropyl- β -phenylethylamine and *o* methyl- β -phenylisopropylamine together with amphetamine have structural and physiological properties in common. None of these compounds have a hydroxyl group on the ring, none of them have an amino group on the terminal carbon atom of the side chain, otherwise they are quite dissimilar except that they are primary amines.

For the present at least one factor in therapeutics, mode of administration, can be correlated very well with the mode of elimination of these compounds. In this particular group of amines the position of the amino group on the side chain determines whether the compound shall be active orally and excreted by the kidneys, or inactive orally and totally inactivated in the body enzymically or otherwise. The compounds that do not have the amino group on the terminal carbon atom are active orally, not because they are not broken down by the digestive juices, but because on being taken into the body they are not deaminated at once when brought by the portal system to the liver. This being the case they are then carried to all parts of the body. Since they remain in the blood stream, at least to some extent, for a long period of time they are cleared from the blood by the kidneys and appear in the urine. Part of the drug remaining in the tissues is inactivated by some system as the ascorbic-dehydroascorbic acid system (5, 13). This, probably together with differences in rate of diffusion and affinity for the tissues, accounts for the differences in rate and extent of excretion of these amines.

Confirming this interpretation, these compounds having an amino group on the terminal carbon atom have no physiological effect when taken orally. Also, they do not appear in the urine as such even when injected subcutaneously. It seems likely then that, administered orally, instead of being broken down in the digestive tract the compound is absorbed, deaminated to some extent in the intestinal wall, where amine oxidase has been shown to be present, and the rest brought to the liver where the enzymic oxidation is completed. Bearing out this point are the experiments showing that even when these compounds, β phenyl-*n*-propylamine and γ phenyl-*n*-propylamine, are injected subcutaneously they are excreted to some extent when the function of the liver is impaired by carbon tetrachloride or hydrazine. These observations point to the liver as being a chief organ for the inactivation of these amines. Amine oxidase and the ascorbic acid system are relatively slow acting when saturated with a proper substrate *in vitro*. It might be that massive doses of one of these amines given orally would partially escape deamination and appear in the urine in very small amounts.

SUMMARY

Sympathomimetic amines having no hydroxyl group on the benzene ring are excreted if the primary or secondary amino group is not on the terminal carbon atom of the side chain, for only in that position is it deaminated by amine oxidase. If the liver function is impaired by CCl_4 or hydrazine, those compounds having a primary or secondary amino group on a terminal carbon atom are also excreted to some extent.

An hypothesis has been presented that the oral efficacy and the excretion of these amines is dependent on whether the compound brought to the liver has its amino group in a position on the side chain where it can be deaminated by the amine oxidase and possibly other systems, normally.

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THE ACTION OF CERTAIN SULFONAMIDES, SULFONES AND RELATED PHOSPHORUS COMPOUNDS IN EXPERIMENTAL TUBERCULOSIS

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Rich and Follis (1) first called attention to the inhibitory action of sulfanilamide for the tuberculous process in guinea pigs. They noted retardation of the progress of infection only if treatment was begun simultaneously with the infection and the drug administered in doses of 1250 mgm. per kilogram per day. Such dosage is at the toxic level, as shown by the death of about 60 per cent of their animals from the toxic effects of the drug within the experimental period of five to six weeks. Later they extended their observations to experimental bovine infection in rabbits and showed that 750 mgm. of the drug given daily per kilogram reduced the extent of tuberculous involvement in the lungs and kidneys, but a dosage of 500 to 600 mgm. per kilogram had no appreciable effect. Significantly 750 mgm. of sulfapyridine per kilogram similarly given had no effect. Examination of the blood at various intervals showed considerably higher blood levels with sulfanilamide than with sulfapyridine (2). Several reports have appeared since then, some confirming the foregoing findings in part or in whole (3-6) and others failing to do so (7-9). Greey, Boddington, and Little (5) obtained a favorable effect in guinea pigs with sulfanilamide but not with prontosil, the dimethyl derivative of disulfanilamide, or with diacetyldiaminodiphenylsulfone. The doses used were within the toxic range, for half of their treated animals died within four weeks of treatment. Corper *et al.* (9) suggest that the apparently favorable effect obtained with large doses of sulfanilamide is due to the toxic effect of the drug and not to the retardation of the tuberculous process.

Further trials with sulfapyridine gave variable results. Feldman and Hinshaw (10) and Birkhaug (11) obtained retardation of the tuberculous process in guinea pigs, while Heise and Steenken (12), Steinbach and Duca (13), Flippin and associates (14), and Muschenheim and coworkers (15) failed to obtain favorable effects. Karlson and Feldman (16) treated rabbits inoculated with avian tubercle bacilli with sulfapyridine with negative results.

An inhibiting action by sulfanilamide on the growth of the tubercle bacillus *in vitro* was reported by Ballou in a concentration of 100 mgm. per cent (17, 18), while Follis could obtain no such effects while using the synthetic Proskauer and Beck medium. Follis was able to demonstrate an inhibiting effect for sulfapyridine in a concentration of 50 mgm. per cent (19).

A much stronger inhibiting action *in vivo* was obtained by Rist, Bloch, and

Hamon (20) for 4-4'-diaminodiphenylsulfone than for sulfanilamide on intravenous avian bacillus infection in rabbits. More recently, while this work was in progress, two very favorable reports have appeared by Feldman, Hinshaw, and Moses (21) on the treatment of experimental tuberculous infection in guinea pigs with the sodium salt of *P-P'*-diaminodiphenylsulfone-*N-N'*-dextrose sulfonate (Promin). In the first report the treated animals survived longer than the controls, and the extent of tuberculous involvement, judged by the amount of tuberculosis in the spleens, was less in the treated animals than in the controls. It was stated, however, that a complicating nutritional disorder developed in the course of the experiment, seemingly an ascorbic acid deficiency, and this may have influenced the results. In a second communication (22) these authors reported equally good results even when treatment with promin was delayed until six weeks after infection. Here again the spleen alone was used to evaluate the extent of tuberculous involvement. It would seem that these workers were dealing with a low grade mild infection, apparently limited for the most part to the spleen and glands. The drug was fed with the diet at a 1 per cent level. Blood examination, they reported, showed an average of 4.3 mgm. per cent of the drug, with a minimum of 2.5 and a maximum of 8.6 mgm. per cent. No toxic effects from the drug were noted in the first report, but sulfhemoglobinemia, reticulocytosis, and possibly anemia are said to have occurred in the second series of experiments. Since promin is a derivative of diaminodiphenylsulfone, a drug which was under study in the work reported here, it seemed desirable to include it in this investigation.

The present experiments were undertaken in the belief that the work cited above indicates sufficiently definitely that the sulfonamides offer enough of a lead to warrant more extensive investigation of the problem. Accordingly, a systematic study was made of the effects of a series of sulfonamides, sulfones, and related phosphorus compounds on the growth of the tubercle bacillus *in vitro*, and the compounds appearing to have good tuberculostatic action *in vitro* were submitted for therapeutic tests in experimental animals. It should be stated at once that neither is the presence of tuberculostatic action of a drug *in vitro* proof for, nor is its absence proof against therapeutic efficiency in experimental animals. Tuberculostatic action, however, may be used as a guide in selecting from a large number of drugs those which are likely to be most promising.

TUBERCULOSTATIC ACTION IN VITRO. The tests for the inhibiting action of drugs on the human tubercle bacillus were made with graded concentrations of the drug in 50 cc. sterile glycerine broth inoculated on the surface with a loopful of a two to three weeks' culture of *H 37*. In several instances the human strain *A 27* was also used with no noticeable difference in results. Pyrex glass Erlenmeyer flasks of 125 cc. capacity were used throughout. The flasks were incubated at 37°C. for two weeks or longer, until the whole surface in the controls was covered with growth. The minimum concentration of the drug which gave only slight or no growth was taken as the effective inhibiting concentration.

The results of this study are shown in table 1. Of the sulfonamides good inhibition was obtained at a level of 20 mgm. per cent with sulfanilyl sulfanilamide

and sulfapyridine, both of which were more active than sulfanilamide. Weight for weight promin was about equally effective. Inhibition at a level of 10 mgm.

TABLE 1
Tuberculostatic action

NUMBER	COMPOUND*	CHEMICAL FORMULA	MGM. PER CENT	
			Good growth	Slight or none
1	Sulfanilic acid	$H_2N \ C_6H_4 \ SO_3OH$	500	
2	Sulfanilamide	$H_2N \ C_6H_4 \ SO_2NH_2$	20	50
3	<i>N'</i> -(4 aminophenyl) sulfanilamide (23)	$H_2N \ C_6H_4SO_2NH \ C_6H_4NH_2$	20	
4	<i>N'</i> -Sulfanilyl sulfanilamide (23)	$H_2N \ C_6H_4 \ SO_2 \ NH \ C_6H_4SO_2NH_2$	10	20
5	Sulfanilylaminoethanol (23)	$H_2N \ C_6H_4SO_2NH \ C_2H_4OH$	100	
6	Sulfanilylamino- β -bromethane ¹	$H_2N \ C_6H_4 \ SO_2NH \ C_2H_4Br$	20	
7	4-Hydroxylaminobenzenesulfonamide ²	$NHOH \ C_6H_4 \ SO_2 \ NH_2$	20	
8	4-Aminobenzoic acid	$H_2N \ C_6H_4 \ COOH$	20	
9	4-Hydroxylaminobenzoic acid ²	$NHOH \ C_6H_4 \ COOH$	20	
10	Sulfapyridine	$H_2N \ C_6H_4 \ SO_2NH \ C_5H_4N$	10	20
11	Sulfathiazole	$H_2N \ C_6H_4 \ SO_2 \ NH \ C_4H_2NS$	1	5
12	Sodium Sulfadiazine ³	$H_2N \ C_6H_4 \ SO_2 \ NH \ C_6H_4 \ N_2$	4	10
13	4-4'-Diaminodiphenylsulfide	$H_2N \ C_6H_4 \ S \ C_6H_4 \ NH_2$	5	10
14	4-4'-Diaminodiphenylsulfoxide	$H_2N \ C_6H_4 \ SO \ C_6H_4 \ NH_2$	1	3
15	4-4'-Diaminodiphenylsulfone ⁴	$H_2N \ C_6H_4 \ SO_2 \ C_6H_4 \ NH_2$	1	2
16	4-4'-Diacetyldiaminodiphenylsulfone ⁵	$CH_3CO \ NH \ C_6H_4 \ SO_2C_6H_4 \ NH \ CO \ CH_3$	10	
17	4-Nitro-4'-acetylamino-diphenyl sulfone (25)	$NO_2 \ C_6H_4 \ SO_2 \ C_6H_4NH \ CO \ CH_3$	10	20
18	Promin ⁶	$[CH_2OH(CHOH)_4CH \ SO_2Na \ NH \ C_6H_4 \ SO_2]$	5	20
19	Phosphanilic acid (26)	$H_2N \ C_6H_4 \ PO(OH)_2$	2	10
20	Dimethylaminophenylphosphonous acid (27)	$(CH_3)_2N \ C_6H_4 \ P(OH)_2$	200	
21	<i>Bis</i> -(dimethylaminophenyl) phosphinous acid (27)	$[(CH_3)_2N \ C_6H_4]_2P \ OH$	20	
22	<i>Bis</i> -(dimethylaminophenyl) phosphinic acid ⁷	$[(CH_3)_2N \ C_6H_4]_2PO \ OH$	10	50
23	<i>Bis</i> -(4'-aminophenyl) phosphinic acid ⁸	$[NH_2 \ C_6H_4]_2PO \ OH$	100	

* Compounds 1, 8, 9, 19, 20, 22, and 23 were used as the sodium salt after careful neutralization with NaOH.

¹ Prepared by Dr. E. L. Jackson of this laboratory.

² Prepared by Dr. H. Bauer of this laboratory by the method of Bamberger and Pyman (24).

³ Courtesy of the Calco Chemical Co.

⁴ Courtesy of the Burroughs Wellcome Co.

⁵ Prepared by Dr. H. Bauer.

⁶ Courtesy of Parke, Davis & Co.

⁷ Prepared by Dr. H. Bauer by the method of Bourneuf (28).

per cent was obtained with 4-4'-diaminodiphenylsulfide and sodium sulfadiazine. The most actively inhibiting compounds were sulfathiazole, which gave good in-

hibition at 5 mgm. per cent, 4-4'-diaminodiphenylsulfoxide at 3 mgm. per cent, and 4-4'-diaminodiphenylsulfone, which inhibited growth at a level of 2 mgm. per cent. Since promin is theoretically about 30 per cent diaminodiphenylsulfone, it is inferior in its tuberculostatic action compared with the parent substance. Substitutions in the amino groups by acetylation or by replacement with nitro groups reduced inhibiting action, while oxidation to the hydroxylamine seems to have had little or no effect. Substitution in the sulfonamide group has resulted in increased activity as in sulfapyridine, sulfathiazole, and sulfadiazine, or in decreased activity as in the instance of sulfanilylaminoethanol.

In the small series of phosphorus compounds phosphanilic acid was the most inhibiting, comparing in activity with 4-4'-diaminodiphenylsulfide and sulfadiazine. This would appear to be interesting for the corresponding sulfur analog, sulfanilic acid, was inactive up to 500 mgm. per cent. It seems probable that with phosphanilic acid as a starting point it may be possible to develop compounds more active than the corresponding sulfur analogs. However, an attempt in this direction represented by compounds 20, 21, 22, and 23 listed in Table 1 failed to attain the goal though, as will be pointed out later, compound No. 21 does appear to have a retarding influence *in vivo* if not *in vitro*. This, we suspect, is due to demethylation in the body to the free amino compound. The lack of activity *in vitro* of compound No. 23, which has free amino groups, does not exclude the possibility that the corresponding trivalent phosphorus compound may prove active. Efforts to prepare this have so far been unsuccessful.

THERAPEUTIC TESTS. From the foregoing list compounds 2, 10, 11, 12, 15, 18, 19, and 21 were selected for therapeutic tests in guinea pigs infected with two strains of human tubercle bacilli and in rabbits infected with a bovine strain.

In the first experiment guinea pigs weighing about 300 grams were divided into five groups each containing twelve animals; all were inoculated intraperitoneally with 0.5 mgm. H 37. For a week prior to inoculation the animals were kept on a diet consisting of 69 per cent ground rolled oats, 25 per cent ground alfalfa, 5 per cent casein, and 1 per cent sodium chloride (29). In addition 25 grams of carrots were allowed per animal per day. Immediately after inoculation the drugs were incorporated in this diet at a level of 0.5 per cent. A record kept of the amount of food consumed indicated an estimated drug intake of from 50 to 75 mgm. per day or about 150 to 300 mgm. per kilogram per day. The experiment lasted 56 days. Whenever an animal died in one of the groups, one animal appearing the sickest in each of the other groups was killed for comparison as to the extent of tuberculous involvement in the several groups. The extent of infection was rated from 0 to 4+ as follows:

+ = Slight. Few miliary tubercles of spleen, liver, omentum, or lungs.

++ = Moderate. Many discrete tubercles in any one of the foregoing organs.

+++ = Moderately advanced. Generalized miliary and conglomerate tubercles in one or more of the organs.

++++ = Advanced caseation necrosis of spleen, liver, omentum, lungs, or all of these.

This strain proved to be of low virulence, the extent of involvement seldom exceeding 2 or 3+, and usually not more than 1+. The infection was usually limited to the omentum, glands, and spleen. From one to six animals died in each group within 25 days subsequent to infection and these are not included in the final analysis. Among those surviving 25 to 56 days, the results were as shown in table 2.

The numerical values for the extent of tuberculous involvement given in the third column

of this table were arrived at by dividing the sum of all the ratings by the number of animals autopsied in each group between the 25th day of the infection and the end of the experiment.

The results of this experiment (table 2) indicate that the drugs were sufficiently toxic to contribute in some measure to the mortality rate, and that none of them in the doses given, except possibly the last-named one, had any beneficial influence in retarding the tuberculous process. There was very little involvement in this group except in the spleens, which were very large, with little or no gross evidence of tubercle formation. Microscopically they usually presented nothing more than epithelioid aggregates or tubercles in early formation with little or no necrosis.

Towards the end of the experiment blood analyses were made in several animals in each group, the determinations having been made in terms of sulfanilamide in each case.¹ As seen in the last column of table 2 this was quite variable and

TABLE 2

Effect of sulfanilamide and related compounds on experimental tuberculous infection in guinea pigs when fed at a level of 0.5 per cent in the diet

GROUP	MORTALITY	AVERAGE EXTENT OF TUBER- CULOSIS	BLOOD LEVELS	
			Free	Total
			<i>mgm. per cent</i>	<i>mgm. per cent</i>
Controls.....	0/10 (0%)	1.4		
Sulfanilamide.....	5/11 (45%)	1.4	Trace-2.3	0.5-6.0
Sulfapyridine.....	8/11 (72%)	1.8	0.4-2.3	1.0-3.5
Sulfathiazole ..	4/6 (66%)	1.7	Trace, 0.4	0.4-0.8
Bis-(dimethylaminophenyl) phos- phinous acid.....	2/10 (20%)	0.3	Trace-0.9	0.5-1.0

generally low. It was obviously difficult by this method of administration to maintain a uniform level of drug intake.

In the second experiment a more virulent strain of tubercle bacilli was used, the animals were kept on a more satisfactory diet of Purina rabbit chow supplemented with cabbage three times a week to supply adequate vitamin C (in which the Purina chow is lacking) and the drugs were administered intragastrically once daily, except Sundays. A No. 8 silk ureteral catheter connected to a syringe was used as a stomach tube. The water-soluble promin and sodium salt of phosphanilic acid were given in aqueous solution, all the others in aqueous suspension with five per cent gum acacia.

In this series there were 96 animals, 16 to a group. The first group served as controls, the second received phosphanilic acid as the sodium salt, the third bis(dimethyl amino phenyl) phosphinous acid, the fourth promin, the fifth diaminodiphenylsulfone, and the sixth sulfadiazine. Promin, sulfadiazine, and phosphanilic acid were given in doses of 0.5 gram per kilogram. This dosage was well tolerated. Diaminodiphenylsulfone was

¹ The method of Bratton and Marshall (J. Biol. Chem., 128: 537, 1939) was used.

given in 0.1 to 0.15 gram per kilogram, and *bis*(dimethyl amino phenyl) phosphinous acid in doses of from 0.2 to 0.5, usually 0.3 gram per kilogram. It seemed desirable to give these drugs to the limits of tolerance. The minimum lethal dose of diaminodiphenylsulfone in guinea pigs on repeated administration was found to be 0.25 gram per kilogram, that of *bis*(dimethyl amino phenyl) phosphinous acid was more variable, the toxic range being between 0.3 and 0.5 gram per kilogram. This drug is more cumulative and less certain in its action. Though a single dose of 0.7 gram per kilogram is usually survived, 0.3 gram per kilogram may prove fatal after repeated daily administrations.

Treatment in this series of experiments was continued for one month. Since all the evidence indicates that the beneficial action of these drugs, if any, is due to inhibition of the multiplication of the tubercle bacillus in the body and a retardation of the tuberculous process possibly through attenuation, it seemed unnecessary to continue treatment beyond the time when dissemination of the tuberculous process is normally well under way. Rather it seemed desirable to give as intensive treatment as possible during the early period of infection.

Accordingly, the animals were infected intraperitoneally with a heavy dose, 0.5 mgm. moist weight, of a virulent human strain of tubercle bacilli A 27.² Treatment was begun on the same day and continued for one month. The animals were weighed once a week and observed carefully for symptoms. At the death of an animal, or when this appeared imminent, the animal was killed and autopsied. A system of rating of the extent of tuberculous involvement, different from that described in the first experiment, was adopted. This consisted in rating the four most commonly involved organs, omentum and glands, spleen, liver, and lungs, from 0 to 4+. One plus designated slight involvement, a few miliary pin point tubercles, 2+ moderate miliary dissemination, 3+ generalized miliary and conglomerate dissemination with occasional patchy caseous necrosis, and 4+ extensive involvement with much caseous necrosis. The sum of these divided by the number of organs gave a numerical value of the extent of tuberculous involvement for each animal. At the end of 107 days when 81 per cent of the controls and 94 per cent in one of the treated groups had died, all the survivors were injected subcutaneously with 0.1 mgm. tuberculo protein (P.P.D.).³ After 24 hours all the survivors were killed and the experiment terminated.

The results of this experiment are summarized in table 3. The comparative mortality rate for the several groups is given at ten-day intervals, and the extent of tuberculous involvement for each animal in each of the groups as well as the average for the whole group is expressed numerically. Examination of the data reveals no favorable effects in the group treated with phosphanilic acid, a definitely retarding influence on the dissemination of the tuberculous process in the group treated with *bis*-(dimethyl amino phenyl) phosphinous acid, and a distinctly favorable influence, both as regards the dissemination of the tuberculous process as well as the survival time, in the groups treated [with promin, sulfadiazine and diaminodiphenylsulfone. The last named drug has, on the whole, appeared to be the most effective. This is indicated by the lowest tuberculosis index and the largest number of survivals following the subcutaneous injection of tuberculin. A comparison of the number of animals showing very slight or no gross tuberculous involvement in these three groups indicates one in the promin series, four in the sulfadiazine, and five in the diaminodiphenylsulfone series.

² This strain was obtained from the Henry Phipps Institute of Philadelphia, through the courtesy of Doctor F. Seibert.

³ Courtesy of Doctor F. Seibert, Henry Phipps Institute of Philadelphia.

TABLE 3

Mortality rate and extent of tuberculous involvement at 10-day intervals

DATE	CONTROLS		PHORPHANILIC ACID		DiS(DIMETHYL AMINOPHENYL) PHOSPHINOUS ACID		PROMIN		DIAMINO-DIPHENYL-SULFONE		SULFADIAZINE	
	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis	Per cent mortality	Tuberculosis
30-39	6	2.5	18	2.0 0.5 1.4	6	0.5	6	1.0	6	2.0	6	1.0
40-49	12	3.0	44	1.7 1.0 3.0 1.7	12	0.5	6		12	1.5	12	1.7
50-59	32	2.2 3.0 1.5	56	2.5 3.0	44	1.0 1.2 1.2 1.5 1.3	12	1.0	25	1.5 1.0	25	2.0 2.2
60-69	50	1.7 2.5 2.0	87	2.5 3.5 2.8 3.2 2.0	56	2.0 1.0	25	0.7 1.0	32	2.2	32	1.2
70-79	69	1.5 1.0 2.0	87		69	2.0 2.4	32	0.7*	32		38	1.7
80-89	81	2.2 3.4	94	2.2	69		38	1.0*	50	1.0* 1.2* 1.0*	44	2.2
90-99	81		94		81	0.8 1.0	44	1.5	56	±	56	±*
107	81	3.2 2.5 2.0	94	2.0	81	1.7† 1.5 1.0	44	1.2 0.2† 1.7 1.5 1.7 2.0 2.0 2.7 1.7	56	0.7 0.7 0.2† 1.5† ±† ±† 0.5†	56	0.5† ±† ±† 1.7 0.7 3.0 2.0
Average tuberculosis index. . .		2.3		2.2		1.3		1.4		0.9		1.4

* Paralysis, killed.

† Same as 0.1 mg. P.D.D. subcutaneous.

More than half of the animals in the last-named group presented an uninterrupted growth curve after cessation of treatment, indicating attenuation of the tubercle bacillus or arrest of the infection.

At from 30 to 60 days, after treatment was discontinued, paralysis of the posterior extremities developed in each of two animals in the promin and sulfadiazine groups and in three animals in the diaminodiphenylsulfone group. It was suspected that the drugs were responsible for what seemed to be a peripheral neuritis. However, careful histological examination of the sciatic nerve and sections of the spinal cord failed to show any lesions. In several of the animals tuberculous infection was found in the meninges in the lumbar region of the spinal cord. It would appear that the paralysees were actually due to tuberculous meningitis. Whether the invasion of the central nervous system is a coincidence, or is in some manner related to treatment, is not possible to say.⁴

TABLE 4
Blood levels, mgm. per cent
(F = free, T = total)

HOURS	PHOSPHANILIC ACID		Bis(DIMETHYLAMINO-PHENYL) PHOSPHINOUS ACID*		PROMIN†		DIAMINODIPHENYL-SULFONE		SULFADIAZINE	
	F	T	F	T	F	T	F	T	F	T
3	0.9	0.9	2.5	2.5	10.5 (3.4)	11.0 (3.5)	4.0	5.5	16.5	15.5
6	0.8	0.8	3.0	3.0	13.5 (4.3)	14.0 (4.3)	4.5	5.0	16.5	17.0
24	0.8	0.8	0.1	0.1	8.0 (2.6)	8.0 (2.6)	3.5	4.5	6.5	6.5

* Estimations made in terms of sulfanilamide, the latter having been used as standard.

† Figures in parentheses are diaminodiphenylsulfone equivalents.

In an attempt to correlate the therapeutic effectiveness of the foregoing drugs with their concentration in the blood a series of normal guinea pigs were treated in the same manner as the infected animals for a period of from 6 to 13 days and the blood levels determined at intervals of from 3 to 24 hours following the last dose. The results of this experiment, expressed as averages of 3 to 4 animals, are shown in table 4. If the drug has some specificity of action, as would appear from its tuberculostatic action *in vitro*, the blood concentration of phosphanilic acid was probably too low to be effective. This and similar experiments in rabbits have shown the poor absorbability of this drug from the gastrointestinal canal. The second phosphorus compound in this series, bis (dimethyl amino phenyl) phosphinous acid, is absorbed with sufficient regularity, and is apparently demethylated to a sufficient degree, to yield a fairly good blood level which is not, however, well maintained despite its cumulative action judged on

⁴ We are indebted to Doctor R. D. Lillie of the Division of Pathology for the histological examination of the tissues.

the basis of toxicological action. The blood level of promin was fairly uniform and well maintained, though perhaps it was not as high as that of diaminodiphenylsulfone when calculated in terms of its equivalent. The somewhat higher and more uniform blood level of diaminodiphenylsulfone, together with its stronger bacteriostatic action *in vitro*, may account for its superior therapeutic effectiveness compared with promin. Under the experimental conditions of treatment the blood level of sulfadiazine was the highest, though it was not as uniform throughout the 24 hours as was the case with the other sulfur compounds. Attention should be called to the low rate of conjugation of this drug compared to the well known high rate of conjugation of sulfanilamide in the guinea pig. This confirms the experiments of Feinstone and associates (30). The lower therapeutic effectiveness of sulfadiazine, despite its higher blood levels compared with diaminodiphenylsulfone, could be explained on the basis of lower specificity as indicated by the relative tuberculostatic actions of these drugs in Table 1.

Two series of experiments with some of the foregoing drugs were also carried out in rabbits.

In these a highly virulent bovine strain of tubercle bacilli, *Ravenel* (S), was used. In the first series 18 rabbits, weighing 2.0 to 3.5 kgm., were each inoculated intravenously with 0.016 mgm. of a fine suspension of tubercle bacilli in 1 cc. sterile salt solution. Six animals served as controls, 6 were treated daily with 0.1 gram per kilogram sodium sulfathiazole and 6 with 0.1 gram per kilogram phosphanilic acid as the sodium salt, both given intravenously. At death the animals were autopsied and the extent of tuberculous involvement noted. This was rated from 0 to 4+ in each of the four organs showing macroscopic lesions, lungs, liver, kidneys, and spleen. The sum of these divided by four gave the "tuberculosis index" for each of the animals, and the sum of these indices divided by the number of animals in the group gave the average index for the group. In the second series of experiments 16 rabbits weighing from 2.0 to 3.0 kgm. were inoculated intravenously with 0.015 mgm. of tubercle bacilli as above. Six served as controls and 10 were treated intravenously daily with 0.3 to 0.5 gram of promin per kilogram, in 10 per cent aqueous solution. Treatment was continued until death, the longest period being 45 days when the last of the controls died. The drug was given in maximum tolerated doses. Doses of 0.3 gram per kilogram usually produced no noticeable effects, while doses of 0.4 and 0.5 gram per kilogram often produced distressing symptoms of dyspnea. Survival time and post mortem findings were noted as in the preceding series.

The results of this study are summarized in tables 5 and 6. Sulfathiazole appeared to inhibit the dissemination of the tuberculous process but had no favorable effect on the survival time. Phosphanilic acid had a more favorable influence. This drug, however, leaves the blood stream rather rapidly, hence it is not possible to maintain an effective blood level with a single daily injection. The rapidity with which the drug leaves the blood stream when injected intravenously or subcutaneously is shown in table 7. The excretion of phosphanilic acid begins soon after injection. The urine turns acid and crystals, apparently of the insoluble acid, appear in abundance. Eighty to 90 per cent of the amount injected is eliminated in the urine within 24 hours, with practically no conjugation.

The toxicity of this compound is low. Rats tolerated one gram per kilogram

TABLE 5

Effect of sulfathiazole and phosphanilic acid in bovine tuberculosis in rabbits

NUMBER	CONTROLS			SODIUM SULFATHIAZOLE			PHOSPHANILIC ACID		
	Weight	Days	Tuberculosis index	Weight	Days	Tuberculosis index	Weight	Days	Tuberculosis index
1	2.0	36	2.5	3.0	19	0.5	2.6	36	1.2
2	3.0	40	2.5	2.4	40	1.5	2.8	46	2.8
3	3.4	46	2.8	2.5	40	2.2	2.9	53	2.0
4	2.2	47	2.3	2.5	49	2.0	2.5	57	1.2
5	2.4	50	2.2	3.7	53	2.5	2.9	77	1.5
6	4.0	53	2.2	3.3	70	2.0	3.1	97	1.3
Average survival...		45.3			45.3			61	
Average tuberculosis index.....			2.4			1.8			1.7

TABLE 6

Effect of promin in experimental bovine infection in rabbits

NUMBER	CONTROLS			TREATED		
	Weight	Days	Tuberculosis index	Weight	Days	Tuberculosis index
1	2.1	21	0.8	2.1	16	0.5
2	2.2	24	2.0	2.1	19	1.2
3	2.3	30	2.5	3.0	24	1.2
4	2.4	31	2.7	2.0	25	1.5
5	2.2	33	2.8	2.5	30	1.2
6	2.3	45	1.5	2.0	30	2.5
7				2.3	39	1.5
8				2.2	53	1.7
9				2.5	74	1.5
10				2.2	122	1.0
Average survival.....		30.7			43.2	
Average tuberculosis index.....			2.1			1.4

TABLE 7

Fate of phosphanilic acid in the rabbit injected as the sodium salt

TIME	INTRAVENOUS INJECTION, 0.3 GRAMS PER KGM.		SUBCUTANEOUS INJECTION, 0.5 GRAMS PER KGM.	
	Blood level			
	Free	Total	Free	Total
5 minutes	110.0	130.0		
1 hour	49.0	50.0	86.0	97.6
2 hours			61.5	86.1
3 hours	13.0	13.2		
4 hours			24.5	25.0
5 hours	4.0	4.2		
6 hours			6.3	6.1
24 hours	Trace	Trace	Trace	Trace

injected intravenously, and in an experiment in a cat under amytal anesthesia a slow intravenous infusion of 2.0 grams per kilogram as a 5 per cent solution of the sodium salt had no effect on the blood pressure or respiration.

The effects of promin seem definite. The survival period in the treated animals was longer, and the extent of tuberculous infection distinctly less. Tuberculous infection was present, however, in every animal; and since the dose used was near the toxic limit, it would seem that promin is less effective in infections with the bovine strain of tubercle bacilli than with the human.

COMMENT. It has been possible to retard the tuberculous process or to check the progress of the disease in experimental animals by means of some of the sulfonamides, sulfones, and certain of the related phosphorus compounds. Rich and Follis (1) were able to do this with sulfanilamide which, however, required toxic doses to achieve the result. In the present study it was possible to accomplish similar results with doses of drugs well within the tolerated range. This suggests greater specificity of some of the drugs we have used. The present experiments also indicate a close parallelism between the effects of the drugs we have used in experimental animals and their tuberculostatic action *in vitro*. All the experiments taken together strongly indicate that diaminodiphenylsulfone is the most effective agent, both *in vitro* and *in vivo*. The toxic nature of this compound and the extreme care with which it has to be administered, coupled with its high degree of specificity, make the search for more effective and less toxic derivatives a promising field of investigation. Diaminodiphenylsulfone appears to have a definitely retarding influence when administered at a level of 50 to 75 per cent of the lethal dose. With the use of this drug as a standard for comparison, the obvious aim is to develop derivatives capable of producing the same or better effects with dosages further removed from the toxic level. Promin does not fulfill this requirement, though it appears to be a step in the right direction.

SUMMARY

A series of sulfonamides, sulfones, and certain related phosphorus compounds were examined for tuberculostatic action *in vitro*. Good inhibition in decreasing order of magnitude was obtained with diaminodiphenylsulfone, diaminodiphenylsulfoxide, sulfathiazole, diaminodiphenylsulfide, sulfadiazine, phosphanilic acid, and promin. Therapeutic tests in experimental animals showed a favorable effect both as regards survival time and retardation of the progress of the disease with diaminodiphenylsulfone, promin, and sulfadiazine. A doubtful effect was obtained with sulfathiazole and *bis* (dimethyl amino phenyl) phosphinous acid, and an irregular result with phosphanilic acid. The last-named drug is poorly absorbed from the gastrointestinal tract, and it leaves the blood stream rather rapidly on intravenous or subcutaneous injection, making the maintenance of a satisfactory blood level difficult.

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STUDIES ON SULFONAMIDE-RESISTANT ORGANISMS

I. DEVELOPMENT OF SULFAPYRIDINE RESISTANCE BY PNEUMOCOCCI

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In our earlier experiments (1, 2) it was noted that under certain conditions sulfanilamide and sulfapyridine inhibited growth of pneumococci for a limited time only. Thus, in *in vivo* experiments (2) in which infected mice were treated with less than curative doses of sulfapyridine, the number of pneumococci in the blood remained stationary at a low level during the first four or five days of treatment; then, notwithstanding continued therapy, the number of organisms increased rapidly and the infection quickly overwhelmed the animals. Observations similar to these were made in *in vitro* experiments (2), in which the growth of pneumococci in sulfapyridine-containing broth was being studied. In these experiments the period of growth inhibition terminated after 24 to 60 hours of incubation. Since this loss in effectiveness of sulfapyridine was not due to a decrease in concentration of the drug, either in the culture medium or the infected animal, it seemed likely that the pneumococci had in some manner adapted themselves to growth in the presence of this drug; in other words, they had become sulfapyridine-resistant or sulfapyridine-fast.

At the time these observations were made there was little information on the phenomenon of sulfonamide resistance. MacLean, Rogers and Fleming (3) had reported that pneumococci acquired resistance to sulfapyridine *in vivo*, but the observed changes in sensitivity were relatively small and not entirely conclusive. MacLeod and Daddi (4) reported development of a sulfapyridine-resistant strain of pneumococcus by an *in vitro* technique, but this report was preliminary and gave no details as to the rate at which resistance developed. In view of the practical and theoretical implications of these observations on sulfapyridine resistance, a more complete and critical study seemed indicated. Such a study was undertaken with attention being paid to the conditions of the *in vivo* and *in vitro* experiments mentioned above. Preliminary reports of this work have been presented heretofore (5, 6); the detailed results are presented here.¹

¹ Since the preliminary reports of this work were presented, Schmith (7) and Lowell, Strauss and Finland (8) published the results of their *in vitro* studies on the development of sulfonamide resistance by pneumococci. The *in vivo* development of such resistance has been reported by Mulder (9) and Schmith (10). Whereas the resistance developed in the experiments of the above workers was not as great as that produced in our experiments, the essential findings in the above publications agree with those reported here.

DEVELOPMENT OF RESISTANCE *in vivo*

A. Method. One strain of type I pneumococcus and two strains of type III were used in this experiment. These strains had been used in our previous studies; consequently, their responses to sulfapyridine were fairly well established. Stock cultures of these organisms were passed repeatedly through mice until constant virulence and invasiveness were attained. Then the organisms were passed serially through groups of sulfapyridine-treated mice in the following manner. Thirty to forty white mice, males, weighing 18 to 22 grams, were infected intraperitoneally, each mouse receiving 10^{-6} cc. of a 12- to 14-hour blood broth culture of the desired organism. Ten of these mice were kept as untreated controls. The remaining animals were treated with sulfapyridine. In the initial experiments, the mice infected with the type I strain received 5 mgm. doses of sulfapyridine, whereas those infected with the type III strains received 20 mgm. doses. These amounts, suspended in 10 per cent acacia, were administered by stomach tube at 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for five additional days. These treatments were based on earlier experiments which showed that 5 mgm. doses in infections with the type I strain and 20 mgm. doses in infections with the type III strains prolonged life for at least six days, but cured no more than one-third of the infected mice.

Cultures of heart blood in infusion broth were prepared from at least 6 treated mice that died between seven and ten days after infection. These cultures were incubated at 37.5°C. for 12 to 14 hours and then pooled; 0.5 cc. of this pooled culture was injected intraperitoneally into an untreated mouse. A 12- to 14-hour blood-broth culture prepared from the heart blood of this mouse was used to infect a second group of mice which received the same sulfapyridine treatment as the first group.

This passage procedure was repeated until the survival time of the treated mice was reduced to such an extent that it approximated the survival time of the untreated controls. Experiments with the type III strains, CHA and Wistuba, were terminated at this point, since in the initial experiments 20 mgm. doses of sulfapyridine were administered. In the experiments with the type I strain, McGovern, where 5 mgm. doses were used initially, additional passages were carried out with the sulfapyridine dosage increased first to 10 and then to 20 mgm. No doses larger than 20 mgm. were used since previous experiments showed that this dosage of sulfapyridine administered at 8-hour intervals had as much curative action as larger doses.

At least two serial experiments, such as the above, were carried out with each of the three strains mentioned. The essential findings in duplicate experiments were identical. The results of typical experiments with each strain have been summarized in tables 1, 2, and 3.

B. Results. The type III strains, CHA and Wistuba, which were naturally slightly resistant to sulfapyridine *in vivo*, acquired a high degree of resistance after as few as 3 serial passages. As table 1 shows, the resistance of strain CHA was increased markedly by the first passage. Thus, mice infected with organisms that had been passed once through sulfapyridine-treated animals (Experiment B) lived on the average only 81 hours, whereas those infected with the parent strain (Experiment A) lived 185 hours. The second and third passages produced a further increase in resistance, so that mice infected with organisms passed three times through sulfapyridine-treated animals (Experiment D) lived only 46 hours—i.e., just 20 hours longer than the untreated controls. This represented the limit of resistance that could be obtained using the procedure described, since three additional passages failed to increase resistance to the drug (cf. Experiments E, F, and G).

In contrast to the result with strain CHA, the first serial passage produced no apparent change in the sensitivity of strain Wistuba (cf. Experiments A and B, table 2). The second passage, however, led to an enormous increase in resist-

TABLE 1

Development of sulfapyridine resistance by type III pneumococcus, strain CHA

EX- PERI- MENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS								SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS	
	Source	Num- ber in in- fecting dose			Days after infection:									Num- ber	Per cent
					1	2	3	4	5	6	7-10				
												hours			
A	From stock after 85 passages	600	30 10	SP20* None	0 7	0 3	0 0	0 0	2 0	5 0	23 0	185 24	0 0	0 0	
B	From 6 exper. A mice, dead on day 7	330	27 10	SP20 None	0 10	1 0	9 0	13 0	2 0	2 0	0 0	81 22	0 0	0 0	
C	From 6 exper. B mice, dead on day 3	300	30 10	SP20 None	0 8	5 2	20 0	5 0	0 0	0 0	0 0	59 24	0 0	0 0	
D	From 10 exper. C mice, dead on day 3	280	30 10	SP20 None	0 4	22 6	8 0	0 0	0 0	0 0	0 0	46 26	0 0	0 0	
E	From 10 exper. D mice, dead on day 2	400	30 10	SP20 None	0 7	21 3	9 0	0 0	0 0	0 0	0 0	43 23	0 0	0 0	
F	From 11 exper. E mice, dead on day 2	300	30 10	SP20 None	0 4	20 6	10 0	0 0	0 0	0 0	0 0	45 26	0 0	0 0	
G	From 8 exper. F mice, dead on day 2	300	30 10	SP20 None	0 7	19 3	10 0	1 0	0 0	0 0	0 0	45 24	0 0	0 0	
H	From stock after 109 passages	300	30 10	SP20 None	0 6	0 4	0 0	0 0	1 0	0 0	29 0	186 24	0 0	0 0	

* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

ance; thus when mice were infected with pneumococci that had been passed twice through treated animals (Experiment C), their average survival time was only 58 hours, as compared with survival times of 173 and 171 hours for mice infected with the parent strain and the organisms recovered from the first serial passage (Experiments A and B). As Experiment D shows, the third serial

passage led to a further increase in the resistance of strain Wistuba. As in the experiment with strain CHA, however, no greater resistance was acquired during two additional passages (Experiments E and F).

More serial passages were required to make type I, strain McGovern, highly resistant than were necessary in the experiments with the type III organisms. This was probably due to the fact that this type I strain was originally much more sensitive to sulfapyridine than either type III strain. However, after 9 serial passages through groups of mice treated with 5, then 10, and finally 20 mgm. doses of sulfapyridine, strain McGovern was as resistant to this drug as were strains CHA and Wistuba (table 3). Comparison of Experiments A and J shows the striking difference in the response of strain McGovern before and after passage through sulfapyridine-treated animals. Thus of the mice infected with the parent strain (Experiment A), 40 per cent of those receiving 5 mgm. doses of sulfapyridine and 83 per cent of those receiving 20 mgm. doses recovered from the infection; the average survival times of the mice that did not recover were 170 and 234 hours for the respective groups. In contrast to this, 5, 10, and 20 mgm. doses of sulfapyridine were entirely without curative action against infections with organisms isolated from the ninth serial passage (Experiment J). In fact, the average survival time of the treated animals, 50 hours, was only 18 hours more than that of the control animals.

It is noteworthy that the resistance of strain McGovern to increasing doses of sulfapyridine developed progressively (cf. Experiments A to I). Nevertheless, when resistance to 20 mgm. doses of this drug was well established, 5 and 10 mgm. doses prolonged life as much as 20 mgm. doses (Experiment J). The explanation for this fact is not clear as yet.

It is particularly important to note the final experiments in tables 1, 2, and 3. These experiments were designed to determine the effect of animal passage on the response to sulfapyridine. They were carried out in the same manner as the first experiment in each table, except that the infecting organisms were the parent organisms that had been passed through untreated mice once daily during the time required to make the different strains resistant. Comparison of the results of these final experiments with those of Experiment A of each table shows conclusively that continued animal passage *per se* was not responsible for the changes in resistance described above.

C. Comparison of the in vitro response of the parent and resistant strains. Since it has been observed (2, 11, 12) that the response of pneumococci *in vivo* does not necessarily parallel their response *in vitro*, it seemed of interest to know whether these organisms that had acquired resistance to sulfapyridine in the preceding *in vivo* experiments were likewise resistant to this drug *in vitro*. Accordingly, a comparative study was made of the growth of the parent and resistant organisms in media containing various concentrations of sulfapyridine.

The medium selected for this work was a beef heart infusion broth (2) enriched with 3 per cent rabbit blood; 50 cc. quantities of this medium, containing 0, 10, and 20 mgm. per cent sulfapyridine, were placed in milk dilution bottles, then

inoculated with 0.5 cc. of a 10^{-4} dilution of a 12-hour culture of the desired organism and incubated at 37.5°C . At various intervals, 0.5 cc. samples of culture were withdrawn, diluted serially and the numbers of organisms in appropriate dilutions were determined by means of blood agar pour plates.

TABLE 2

Development of sulfapyridine resistance by type III pneumococcus, strain wistuba

EX- PERI- MENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS								SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS	
	Source	Num- ber in in- fecting dose			Days after infection:									Num- ber	Per cent
					1	2	3	4	5	6	7-10				
A	From stock after 156 passages	500	20	SP20*	0	0	0	0	0	1	15	173	4	20	
			10	None	7	3	0	0	0	0	0	25	0	0	
B	From 7 exper. A mice, dead on day 8	560	30	SP20	0	0	0	0	0	1	28	171	1	3	
			10	None	5	5	0	0	0	0	0	25	0	0	
C	From 6 exper. B mice, dead on day 7	900	30	SP20	0	4	24	1	0	1	0	58	0	0	
			10	None	8	2	0	0	0	0	0	25	0	0	
D	From 8 exper. C mice, dead on day 3	280	30	SP20	0	24	6	0	0	0	0	43	0	0	
			10	None	8	2	0	0	0	0	0	22	0	0	
E	From 9 exper. D mice, dead on day 2	890	30	SP20	0	16	13	1	0	0	0	49	0	0	
			10	None	6	4	0	0	0	0	0	23	0	0	
F	From 6 exper. E mice, dead on day 2	800	30	SP20	0	21	9	0	0	0	0	44	0	0	
			10	None	6	4	0	0	0	0	0	25	0	0	
G	From stock after 192 passages	500	30	SP20	0	0	0	0	2	1	17	166	10	33	
			10	None	5	5	0	0	0	0	0	25	0	0	

* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

The results of typical experiments are shown in figure 1. These data show that the resistant strains of type I McGovern and type III Wistuba multiplied as rapidly in broth containing 20 mgm. per cent sulfapyridine as in control broth. Growth of the resistant strain of type III CHA was inhibited slightly by 20 mgm. per cent sulfapyridine, but not at all by 10 mgm. per cent. Contrasted with this are the results with the parent organisms, growth of which was in-

TABLE 3

Development of sulfapyridine resistance by type I pneumococcus, strain McGovern

EX- PERI- MENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS								SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS	
	Source	Num- ber in infect- ing dose			Days after infection:									Num- ber	Per cent
					1	2	3	4	5	6	7- 10				
A	From stock after 290 passages	380	30	SP5*	0	0	0	0	1	8	9	170	12	40	
			30	SP20*	0	0	0	0	0	0	5	234	25	83	
			10	None	1	9	0	0	0	0	0	30	0	0	
B	From 8 exper. A mice, dead on day 6	740	30	SP5	0	0	0	2	2	6	14	155	6	20	
			10	None	0	10	0	0	0	0	0	33	0	0	
C	From 8 exper. B mice, dead on day 3	680	30	SP5	0	2	16	7	2	3	0	72	0	0	
			10	None	0	10	0	0	0	0	0	28	0	0	
D	From 9 exper. C mice, dead on day 3	1280	30	SP10*	0	1	9	4	7	1	5	98	3	10	
			10	None	0	10	0	0	0	0	0	37	0	0	
E	From 8 exper. D mice, dead on day 3	550	30	SP10	0	0	4	2	12	2	10	124	0	0	
			10	None	2	8	0	0	0	0	0	38	0	0	
F	From 9 exper. E mice, dead on day 5	1150	30	SP10	0	2	11	6	2	3	6	104	0	0	
			10	None	0	10	0	0	0	0	0	37	0	0	
G	From 8 exper. F mice, dead on day 3	550	30	SP10	0	18	10	2	0	0	0	53	0	0	
			10	None	0	10	0	0	0	0	0	35	0	0	
H	From 12 exper. G mice, dead on day 2	640	30	SP20	0	11	13	6	0	0	0	57	0	0	
			10	None	0	10	0	0	0	0	0	34	0	0	
I	From 12 exper. H mice, dead on day 2	520	30	SP20	0	5	23	2	0	0	0	58	0	0	
			10	None	0	10	0	0	0	0	0	38	0	0	
J	From 12 exper. I mice, dead on day 2	440	30	SP5	1	18	8	3	0	0	0	49	0	0	
			30	SP10	3	12	13	2	0	0	0	50	0	0	
			30	SP20	0	15	15	0	0	0	0	50	0	0	
			10	None	0	10	0	0	0	0	0	32	0	0	
K	From stock after 330 passages	450	30	SP5	0	0	0	0	0	2	8	171	20	67	
			30	SP10	0	0	0	0	2	0	2	174	26	87	
			30	SP20	0	0	0	0	0	0	2	189.5	28	93	
			10	None	0	10	0	0	0	0	0	36	0	0	

hours after infection

hibited markedly by 10 mgm. per cent of the drug. It is evident, therefore, that these strains that had been made resistant *in vivo* were likewise resistant *in vitro*.

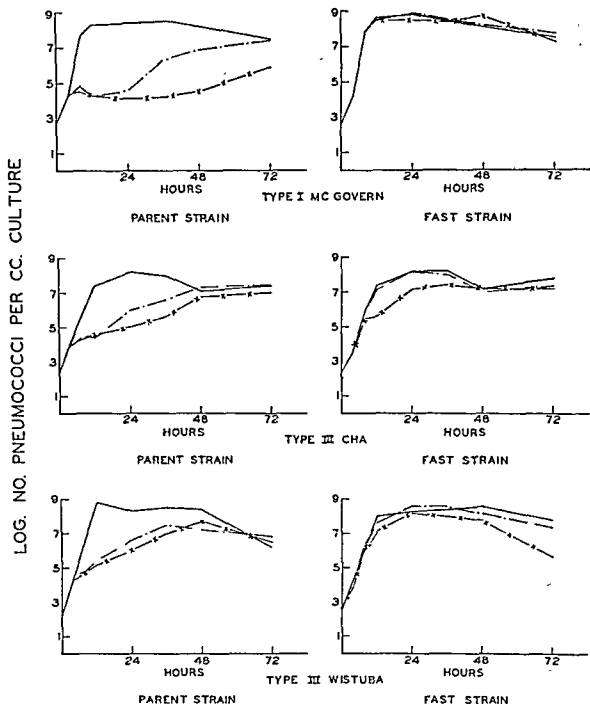


FIG. 1. EFFECT OF SULFAPYRIDINE ON GROWTH OF PARENT AND RESISTANT STRAINS OF TYPE I PNEUMOCOCCUS MCGOVERN AND TYPE III PNEUMOCOCCI CHA AND WISTUBA

—, growth in beef heart control media; ---, growth in beef heart media containing 10 mgm. per cent sulfapyridine; —X—, growth in beef heart media containing 20 mgm. per cent sulfapyridine.

DEVELOPMENT OF RESISTANCE *in vitro*

A. Method. A type II pneumococcus, strain CH, was used in these experiments. This organism had undergone nearly 100 consecutive daily mouse passages prior to use and was selected for the present study on the basis of earlier experiments (2) which showed that it

was highly sensitive to sulfapyridine *in vitro*. The following procedure was used to make this organism highly resistant to the drug. Fifty cubic centimeter quantities of beef heart infusion broth, containing 0, 4, 8, and 15 mgm. per cent sulfapyridine, were placed in milk dilution bottles. Each quantity of medium was enriched with 1.5 cc. of rabbit blood, inoculated with 0.5 cc. of a 10^{-4} dilution of a 12-hour blood broth culture of the parent organism, and then incubated at 37.5°C . At various intervals 0.5 cc. samples of the cultures were withdrawn, diluted serially, and the numbers of organisms in appropriate dilutions determined by means of blood agar pour plates. When growth had occurred in the culture containing 8 mgm. per cent sulfapyridine, the organisms from this culture were subcultured into plain beef heart infusion broth enriched with blood. This latter culture was incubated for 12 hours, then used as the source of organisms for a second experiment, otherwise identical with the first. This general procedure was repeated in 5 additional experiments; in these, however, the sulfapyridine content of the medium was increased gradually, until growth was obtained in media containing 160 mg. per cent of the drug ("saturated" media as shown in chart 7 of fig. 2).

In order to determine whether prolonged culture in artificial media in itself altered the sensitivity of strain CH to sulfapyridine, the following experiment was carried out simultaneously with the one just described. The parent organism was cultured in plain beef heart infusion broth, enriched with blood, for the same time and subcultured at the same intervals as the organisms that were transferred through sulfapyridine-containing broth. At the conclusion of these passages in plain broth, the sensitivity of this parent organism was studied, using a procedure identical with that described in the first test above.

B. Results. Charts 1 to 7 of figure 2 show graphically the changes in sensitivity which occurred as type II, strain CH, was passed serially through media containing increasing concentrations of sulfapyridine. The first experiment (Chart 1) shows the response of the parent organism. Growth of this organism was inhibited slightly in media containing 4 mgm. per cent sulfapyridine and was checked completely by 15 mgm. per cent of the drug. Growth in media containing 8 mgm. per cent sulfapyridine was inhibited almost completely for nearly 48 hours, then slow multiplication occurred. The result obtained after 6 serial passages of this organism through sulfapyridine-containing media is in marked contrast to that just described. After this number of passages (Chart 7), growth was nearly as rapid in media containing 90 and 160 mgm. per cent sulfapyridine as in control media. The results in Charts 2 to 6 indicate that this change in sensitivity was brought about progressively, with a small but definite increase in resistance occurring even during the initial passage through the sulfapyridine-containing media. The results shown in Charts 4 and 5 are particularly indicative of a progressive development of resistance. Thus, in the experiment shown in Chart 4, the concentrations of sulfapyridine were increased too much over those used previously, with the result that growth failed to occur in the presence of higher drug concentrations than those used in the preceding experiment. In the next experiment, however (Chart 5), the concentrations of sulfapyridine were raised more gradually, with the result that growth occurred in a higher concentration of drug than was used previously.

The results of the control experiment are shown in Chart 8 of figure 2. These data show conclusively that repeated culture of the parent organism in plain beef heart infusion broth was not in itself responsible for the increase in resistance noted above. In fact, the natural resistance of the parent organism seemed to

LOG NO. OF PNEUMOCOCCI PER CC. CULTURE

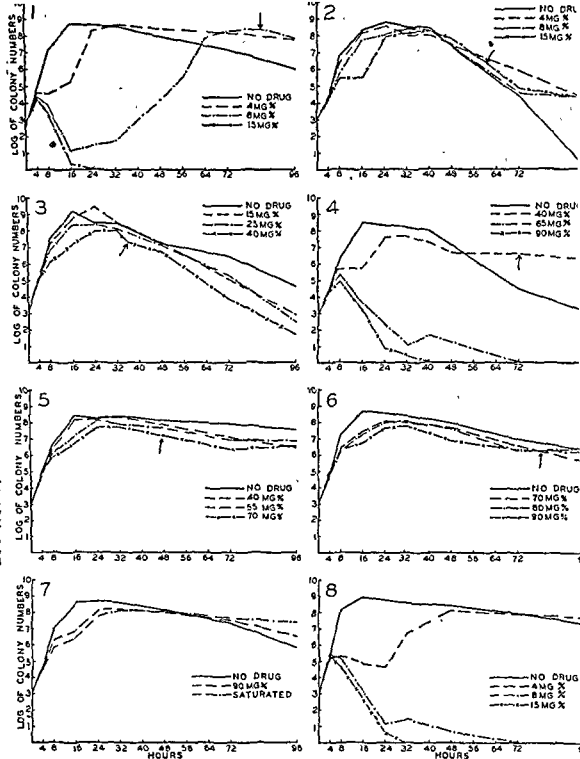


FIG. 2. DEVELOPMENT OF SULFAPYRIDINE RESISTANCE IN TYPE II PNEUMOCOCCUS, STRAIN CH

of organisms isolated from second serial
of organisms isolated from third serial

be decreased slightly following such treatment, since the passaged strain failed to grow in media containing 8 mgm. per cent sulfapyridine, whereas the original parent organism had been able to multiply.

C. *Comparison of the in vivo response of the parent and resistant organism.* It seemed of interest to know whether the organisms isolated from the media saturated with sulfapyridine (Chart 7) were resistant to this drug *in vivo*. Before

TABLE 4
Retention of resistance by type II pneumococcus, strain CH

EX- PERI- MENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS								SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS	
	Source	Num- ber in infect- ing dose			Days after infection:									Num- ber	Per cent
					1	2	3	4	5	6	7-10				
A	Parent organism after 130 mouse passages	250	30 10	SP20* None	0 0	0 10	0 0	0 0	0 0	0 0	19 0	hours 184 30	11 0	37 0	
B†	From <i>in vitro</i> ex- per. 7 after 12 mouse passages	1510	30 10	SP20 None	0 1	29 9	1 0	0 0	0 0	0 0	0 0	38 27	0 0	0 0	
C‡	From <i>in vitro</i> ex- per. 7 after 90 mouse passages	200	30 10	SP20 None	0 0	16 10	14 0	0 0	0 0	0 0	0 0	48 38	0 0	0 0	
D	From <i>in vitro</i> ex- per. 7 after 169 mouse passages	300	20 10	SP20 None	0 2	20 8	0 0	0 0	0 0	0 0	0 0	41 29	0 0	0 0	
E	From <i>in vitro</i> ex- per. 7 after 223 mouse passages	280	30 10	SP20 None	0 1	30 8	0 1	0 0	0 0	0 0	0 0	38 31	0 0	0 0	

* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

† Organisms used in this experiment were passaged through untreated mice and cultured in media containing 160 mgm. per cent sulfapyridine for all 12 passages.

‡ Organisms used in this and the subsequent experiments were passaged through untreated mice and cultured in media containing no sulfapyridine.

the *in vivo* test could be carried out, however, the mouse virulence of this resistant strain had to be restored. This was accomplished by 12 passages of the organism through normal untreated mice, the blood of these passage mice being cultured each time in media saturated with sulfapyridine. The *in vivo* response of this organism to sulfapyridine was then studied, the infection and treatment procedure being identical with that used in the *in vivo* experiments described earlier in this report. A similar experiment with the parent type II strain was carried out simultaneously.

The results of this study (Experiments A and B, table 4) show that the organism that had been made sulfapyridine-resistant *in vitro* was highly resistant *in vivo*.² The comparison between the responses of the resistant and parent strains is striking. None of the mice infected with the resistant organism recovered and the average survival time of such mice was only 38 hours. On the

TABLE 5
Retention of resistance by type III pneumococcus, strain CHA

EX- PERI- MENT	ORGANISMS		NUM- BER OF MICE IN- FECTED	TREAT- MENT	NUMBER OF DEATHS								SUR- VIVAL OF MICE THAT DIED	THIRTY-DAY SURVIVORS	
	Source	Num- ber in in- fecting dose			Days after infection:									Num- ber	Per cent
					1	2	3	4	5	6	7- 10				
A	From exper. F (table 1) after 26 mouse pas- sages	400	30	SP20*	0	19	9	2	0	0	0	47	0	0	
			10	None	8	2	0	0	0	0	0	23	0	0	
B	From exper. F (table 1) after 44 mouse pas- sages	480	30	SP20	0	14	15	1	0	0	0	48	0	0	
			10	None	6	4	0	0	0	0	0	26	0	0	
C	From exper. F (table 1) after 73 mouse pas- sages	750	20	SP20	0	11	8	1	0	0	0	47	0	0	
			10	None	6	4	0	0	0	0	0	24	0	0	
D	From exper. F (table 1) after 142 mouse pas- sages	740	25	SP20	0	10	14	1	0	0	0	51	0	0	
			10	None	5	5	0	0	0	0	0	25	0	0	
E	From exper. F (table 1) after 215 mouse pas- sages	1200	30	SP20	1	19	10	0	0	0	0	45	0	0	
			10	None	8	2	0	0	0	0	0	24	0	0	

* SP20 = 20 mgm. sulfapyridine 2, 8, 14, and 22 hours after infection and every 8 hours thereafter for 5 days, or as long as the animals survive.

other hand, 11 of the mice infected with the parent organism recovered, and the average survival time of the remainder was 184 hours. It should be noted that

² It should be pointed out that pneumococci that have been made sulfapyridine-resistant *in vitro* are not always resistant *in vivo*. Thus Sesler and Schmidt (12) have found that 3 strains of pneumococci, which had been made highly resistant to sulfapyridine, sulfathiazole and sulfanilamide by an *in vitro* technique somewhat different than that described above, were only slightly more resistant to these drugs *in vivo* than were the parent organisms.

these strains were about equally invasive, the death times of untreated mice being essentially the same.

RETENTION OF RESISTANCE. It seemed of considerable interest to know how long the above strains retained their resistance to sulfapyridine. Accordingly, the various resistant strains were passed repeatedly through untreated mice, the blood of these animals being cultured at each passage in infusion broth containing no drug. At various intervals the *in vivo* responses of these organisms were tested by means of the procedures outlined in the previous experiments.

All 4 resistant strains, type I McGovern, type II CH, and type III, Wistuba and CHA, reacted in a similar manner; consequently only the results with the type II strain made resistant *in vitro*, and with the type III CHA strain made resistant *in vivo*, are presented here. As the data in tables 4 and 5 show, passage of type II, strain CH, through 223 normal mice and type III, strain CHA, through 215 mice did not alter the resistance of these strains to sulfapyridine. Thus it must be concluded that sulfonamide resistance is a characteristic which is retained almost indefinitely once it is well established. However, as a previous study has shown (5), resistance can be lost when this characteristic is only partially developed.

Discussion. As has been mentioned previously, our earlier experiments (1, 2) showed that under certain conditions sulfanilamide and sulfapyridine inhibit growth of pneumococci for a limited time only. Other investigators, working with pneumococci (10, 13), streptococci (14) and *Brucella* (15), have observed this same phenomenon in varying degree. Green (15) attributed the late outgrowth of *Brucella* to an increase in the amount of a growth-stimulating substance termed "P" factor. The data in the present experiments show clearly, however, that the late outgrowth of pneumococci is due primarily to the development of sulfonamide-resistant organisms, rather than to a change in the composition of the medium.

One of the first questions raised by the present study concerns the mechanism involved in converting sulfonamide-sensitive strains of pneumococcus into resistant strains. The current experiments have shown that the development of resistance is a progressive process, and that when resistance is well established it is retained by innumerable succeeding generations of organisms. Dubos (16) has pointed to these phenomena as characteristics of a process of selection. It seems probable, therefore, that the resistant strains are developed by selective propagation of sulfonamide-resistant variants.

How these resistant variants are formed originally is a question that cannot be answered with the data available. However, judging from experiments on the formation of yeast variants (17), one may suggest that sulfonamide-resistant variants may be formed either during normal multiplication of the sensitive parent organisms, or in response to some injurious action of the sulfonamide. There is a certain amount of evidence that can be interpreted as supporting each of these possibilities. Thus, Frisch (18) observed that pneumococci obtained from a single sputum sample of an untreated pneumonia patient exhibit varying degrees of resistance to the sulfonamides. This observation may indicate that

organisms possessing varying degrees of resistance are formed during normal multiplication of pneumococci. On the other hand, McKinney and Mellon (19) found that pneumococci exposed to sulfonamides do undergo dissociative changes, and Beall (20) reported recently that exposure to sulfanilamide alters the chromosomal arrangement of certain plants; these observations suggest that sulfonamides may induce mutations, and it is possible that a sulfonamide-resistant organism may be one of the mutants formed. It should be pointed out, however, that the process of selection following appearance of the first resistant variants would probably be the same, regardless of the manner in which the variants are formed.

It may be questioned whether the resistant variants formed directly from the sensitive parent organisms are fully resistant to sulfapyridine. The results of the experiment with type II, strain CH (fig. 2), suggest that these first variants are only mildly resistant. When these mildly resistant variants are exposed to concentrations of sulfapyridine that inhibit their growth, they give rise to more highly resistant organisms, just as did the parent pneumococci. By repeating this process it is possible to eliminate sulfonamide-sensitive organisms entirely and to obtain a culture made up exclusively, or almost exclusively, of organisms that can multiply rapidly in the highest concentrations of sulfapyridine that can be maintained in the body fluids of animals or in artificial media.

It is noteworthy that the superficial properties of the resistant and sensitive organisms studied in this laboratory seem to be identical. These properties include morphology, capsule formation, type specificity, inulin fermentation, bile solubility, growth on blood agar, growth rates in beef heart infusion broth, and virulence and invasiveness for mice.

The above observations raise the question as to what characteristics of the resistant and sensitive organisms determine their responses to sulfonamides. This question cannot be answered satisfactorily at present. It may be suggested, however, that sulfonamide resistance might well be related to any one of the following properties: (1) *Capacity of the organisms to inactivate the sulfonamides.* Inactivation of sulfonamide might be due either to the conversion of the drug into an inactive derivative (perhaps similar to the acetyl form) or to the production of sulfonamide-inhibiting substances like *p*-amino-benzoic acid. There is little information on the production of inactive sulfonamide derivatives by bacteria. On the other hand, sulfonamide-inhibiting substances have been found in a variety of bacteria and yeasts (15, 21, 22); and it is pertinent that MacLeod (23) found more sulfonamide inhibitor in the culture supernatant of a resistant type I pneumococcus than in similar material from a sensitive type I strain. (2) *Capacity of the organism to convert the natural sulfonamide into its hypothetical "active" form.* Several investigators (24-27) have suggested that the active principles of the sulfonamides are the oxidation products rather than the "natural" forms. This has not been demonstrated conclusively, but if it were true, then those organisms that were unable to bring about this oxidation would be sulfonamide-resistant. (3) *Growth requirements of the organism.* Assuming that the sulfonamides inhibit growth by preventing utilization of an

essential growth material in the culture medium (22), then organisms that are sulfonamide-resistant must be able either to synthesize this growth essential, or to utilize some substitute material whose utilization is not blocked by the sulfonamides. (4) *The intermediary metabolism of the organism.* If sulfonamides inhibit growth by blocking an essential metabolic process, then the resistant organism must have a different mechanism for carrying out this reaction than the sensitive organism. MacLeod's observations (28) have suggested that there are differences in the intermediary metabolism of resistant and sensitive strains. According to this investigator, both strains dehydrogenate glucose at equal rates, but the sensitive organisms dehydrogenate lactate, glycerol and pyruvate, whereas the resistant strains do not do this. In this connection it is noteworthy that sulfapyridine interfered with the dehydrogenation of the 3 carbon atom compounds but not with that of glucose.

Critical studies of these various possibilities are now in progress. It may be pointed out that an explanation for sulfonamide resistance might assist materially in understanding the mode of action of sulfonamide drugs.

In conclusion, it should be pointed out that the finding that pneumococci can acquire resistance to sulfapyridine has practical as well as theoretical implications. Studies in pneumococcal pneumonia (8, 29) and pneumococcal meningitis (30) have indicated that the resistance of pneumococci to sulfonamides does increase sometimes during the clinical use of these drugs. Although this problem has not been serious as yet, it seems likely to assume greater importance when these resistant organisms become disseminated by their carriers³. This may curtail the value of sulfonamide therapy seriously—especially since pneumococci that have become resistant to one sulfonamide, such as sulfapyridine, are resistant also to other sulfonamides such as sulfathiazole, sulfamethylthiazole, and sulfadiazine (8, 31).

SUMMARY

(1) One strain of type I and two strains of type III pneumococcus were made highly resistant to sulfapyridine by serial passage through mice treated with less than curative doses of this drug. The organisms that were made resistant *in vivo* were also resistant to sulfapyridine *in vitro*.

(2) A sulfapyridine-sensitive strain of type II pneumococcus was made highly resistant to this drug by serial passage through broth containing increasing concentrations of sulfapyridine. This resistant organism was also insensitive *in vivo*.

(3) Sulfapyridine resistance was retained by the above strains for more than 200 passages through untreated mice.

(4) The manner in which resistant strains are developed and possible explanations for sulfonamide resistance were mentioned and discussed.

³ Sulfonamide-resistant pneumococci have been isolated from nose and throat cultures of patients as long as four months after clinical cure of pneumococcal pneumonia (29).

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THE ISOLATION OF A PROTEIN FROM THE PARS NEURALIS OF THE OX PITUITARY WITH CONSTANT OXYTOMIC, PRESSOR AND DIURESIS-INHIBITING ACTIVITIES¹

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Following the discovery of the pressor, oxytomic, and renal effects of posterior pituitary extracts, there has been considerable controversy with regard to the number of substances responsible for these effects. Abel and his collaborators (1, 2) maintained that the actual hormone is a "unitary" substance containing all the activities whereas Dudley (3) early showed that at least partial separation of oxytomic and vasopressor activities could be effected. After Kamm and his colleagues (4) had achieved practically complete separation of these two activities, there was general acceptance of the view that at least two active principles (oxytomic and vasopressor) occur separately in the *pars neuralis*. This view was further strengthened by confirmatory experiments by other methods such as those devised in Stehle's laboratory (5, 6). However, Abel (2) did not withdraw his contention that the true hormone is one substance but did admit that two active and separable principles could be extracted from the gland by appropriately drastic procedures. Rosenfeld's (7) ultracentrifugation of chilled and clarified press-juice of ox posterior lobes also supported the view that oxytomic and vasopressor activities are linked together in a single fairly large molecule and may be liberated from the more complex unit by heating at about pH 4.0.

The experiments reported here are believed to demonstrate that a pure protein of the *pars neuralis* can be isolated containing constant amounts of oxytomic, pressor, and diuresis-inhibiting activities in ratios resembling those found in simple extracts of the gland. In the discussion, possible objections to this belief will be considered with regard to both the physico-chemical (e.g. the possible rôle of adsorption) and the physiological aspects of the problem.

METHODS. *Physico-chemical methods.* All determinations of pH were made by means of a glass electrode and were accurate to ± 0.02 pH. The determinations of nitrogen, except those of table 2 for which a micro-Dumas method was employed, were all made by a micro-Kjeldahl method by which as little as 100 micrograms of N could be determined with an accuracy of ± 2 micrograms. In addition to micro-determination of total sulfur, sulfur

¹ A preliminary report was published in the Proceedings of the American Physiological Society (Amer. J. Physiol., 133: 473, 1941).

distribution (cysteine, cystine, methionine, and sulfate) was investigated by the Kassel and Brand modification of the Baernstein method (8). Anson's method (9) was used to detect sulfhydryl groups (cysteine). Solubility determinations were made at room temperature. The electrophoretic characteristics of the protein were determined in the apparatus of Tiselius (10); the electrophoretic patterns were photographed by the method of Longworth (11). Ultracentrifugation of solutions of the protein was performed in a Bauer and Pickels type (12) of ultracentrifuge driven by an air turbine of the turret type. The rate of sedimentation was calculated from "schlieren" patterns photographed by Philpot's method as modified by Svensson (13). In several experiments ultracentrifugation of solutions of the protein in separation cells was performed.

Biological methods. Estimates of biological activity were all made in terms of U.S.P. reference standard of posterior pituitary powder. Oxytocic activity of various fractions was determined by two methods: the response of the isolated guinea pig uterus or the depressor effect on the fowl's blood pressure. The "physiological" solution used for the isolated uterus was that of van Dyke and Hastings modified to contain 0.75 mM of Ca per liter (14). Furthermore no Mg was added to the fluid since this cation has been found to increase the uterine response to the vasopressor principle (15). In determining oxytocic action by the depressor effect of extracts on the fowl's blood pressure, the method of Coon (16) was largely followed. Dogs and less frequently cats were employed to estimate the vasopressor activity of extracts. Doses were kept small and repeated at sufficiently infrequent intervals to avoid tachyphylaxis. All determinations of blood pressure in the fowl, dog, and cat were made with the glass capsule manometer of Anderson (17). Clotting in cannulas was prevented by the use of a compact modification of Trendelenburg's apparatus (18) to infuse slowly isotonic saline containing 15 mgm. per cent of heparin. To anesthetize cats or chickens 200 mgm. of phenobarbital sodium per kilogram body weight were injected intraperitoneally (cat) or intramuscularly (fowl); dogs were anesthetized by the intraperitoneal injection of 1 ml. per kilogram of 40 per cent alcoholic solution of chlorbutanol as recommended by Kamm and his co-workers (4).

In estimating the inhibition of diuresis in rats we modified Burn's method (19) only to the extent of administering fluid (0.3 per cent NaCl) intraperitoneally instead of by mouth. As a rule either 28 or 32 rats (7 or 8 groups) were used at one time. Half of the groups received the standard and half received the extract to be assayed; several days later the same doses of the same standard or extract were administered in reverse order. Therefore, comparison of the extract and standard was made in the same groups of rats (7 or 8 groups of 4 rats each) as nearly simultaneously as possible.

The melanosome-dispersing action of extracts was compared with U.S.P. reference standard in frogs. For this purpose, normal frogs or frogs hypophysectomized by the method of Teague, Noojin, and Geiling (20) were used.

THE PREPARATION OF THE PURE PROTEIN FROM EXTRACT OF POSTERIOR LOBES OF OXEN. A diagram of the method of preparation is reproduced in figure 1. The initial suspension of freshly dissected posterior lobes obtained from frozen pituitaries contains 1 kgm. of tissue in 9 l. of cold 0.01 *N* H₂SO₄. The pH of the suspending liquid varies little (about ± 0.1 pH) from the value given if different batches are compared. After the mixture has been thoroughly stirred by an electric motor it is allowed to stand in a refrigerator (4°C.) overnight. The separation of the residue and of all subsequent precipitates from supernatants is accomplished by centrifugation. Precipitation of active protein by the addition of 80 grams NaCl to each liter of supernatant adjusted to pH 3.90 (step 2) is also allowed to continue overnight at 4°C.

The last step by which complete purification is achieved is repeated until

solubility is constant. This step is carried out at room temperature which probably is an important variable (20 to 25°C.) affecting solubility. In this final step, constant solubility rather than a solubility of exactly 100 micrograms of N per ml. is sought. In our experience the apparently pure protein may have a solubility as low as 80 micrograms of N per ml.; however, this solubility is constant in a solvent made as exactly as possible like that described in figure 1 in the step next to the last.

The method of extraction is not of high efficiency in terms of the total activity available (about 200,000 units per kg. fresh posterior lobes). The residue

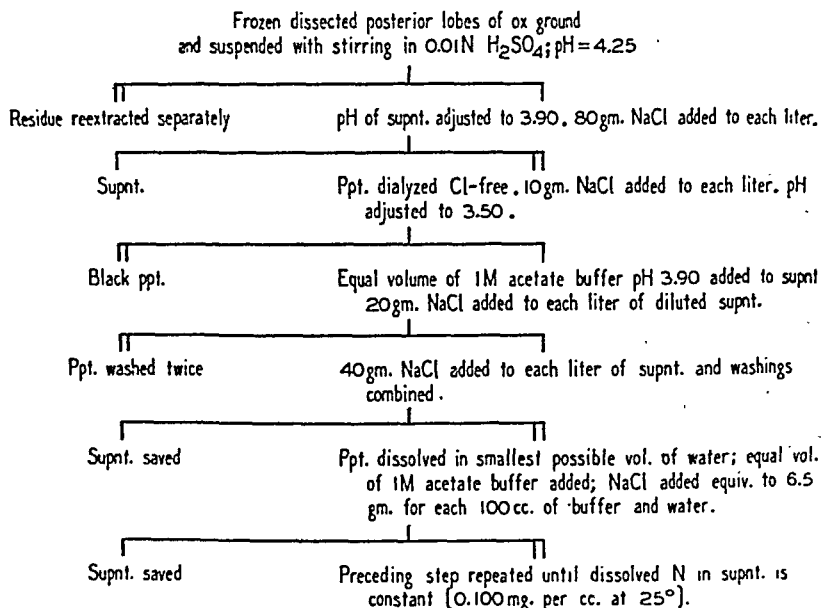


FIG. 1. METHOD OF PREPARING A PURE PROTEIN WITH MULTIPLE ACTIVITIES BY EXTRACTION OF FRESH POSTERIOR PITUITARIES OF OXEN

after initial extraction contains 20 to 25 per cent of total activity which can be removed by boiling a sample of the ground dissected lobes in 0.25 per cent acetic acid. About two-thirds of the 75 to 80 per cent total activity in the supernatant liquid separated from the residue is precipitated by the addition of 80 grams of NaCl to each liter. Therefore, about 50 per cent of total activity is present in the precipitate at the end of the second step. During dialysis of this precipitate activity which apparently is non-protein is also lost; however, we have not attempted to estimate this loss accurately. From 1 kgm. of fresh glands about 700 mgm. of pure protein (>11,000 units) can be isolated apart from subsequent recoveries in supernatants of the last two steps. Samples of the pure protein so far isolated are amorphous.

EVIDENCE THAT THE PROTEIN ISOLATED IS PURE. *Constant solubility.* Northrop and his collaborators (21) in studying crystalline enzymes have applied with great success the solubility test of Sørensen in determining the presence of small amounts of impurity or in demonstrating homogeneity of crystalline enzymes. The solubility characteristics of the protein isolated in the example of figure 1 are shown in figure 2. The solvent used was 0.5 M acetate buffer, pH 3.90, to which 6.5 grams of NaCl were added to each 100 ml. It is clear that by this test there is no evidence that more than one component is present either before the solvent is saturated with the protein or after twenty times the saturating concentration is in suspension. The amounts of protein which have been available have not permitted us to carry out solubility tests in other solvents which should be employed. We have already mentioned that at different times or with other preparations, constant solubility in the same solvent made later might be as low as 0.080 mgm. of N dissolved in each ml. Variables such as

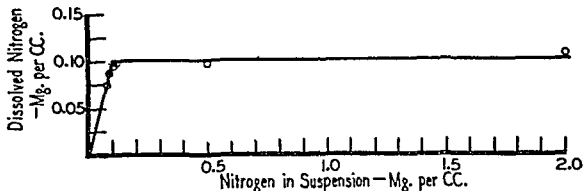


FIG. 2. SOLUBILITY OF THE PURE PROTEIN IN 0.5 M ACETATE BUFFER, pH = 3.90, TO WHICH 6.5 GRAMS NaCl WERE ADDED TO EACH 100 ML.

room temperature or slight changes in the pH of the buffer (e.g., pH 3.95 instead of 3.90) probably explain the variation in absolute amount of protein dissolved.

Electrophoretic homogeneity. A large number of electrophoretic patterns of the pure protein have been photographed in the Tiselius electrophoresis apparatus. In figure 3, mobility per centimeter per second per volt per centimeter has been plotted against pH. The isoelectric point of the protein appears to be about pH 4.8. Examples of patterns photographed by the method of Longworth (11) are shown in figure 4. The upper three patterns were made with three different preparations of pure protein at pH 3.41-3.47. At this pH only, of those used (fig. 3), there appears to be a second protein which is indeed small in comparison with the main component. The oxytocic activity of this minor substance is no greater than that of the main component in terms of nitrogen. At other pHs (e.g., preparation XI-85-G at pH 6.05) there is electrical inhomogeneity but no second protein can be separated. It appears that the minor component at pH 3.4-3.5 is closely related to the main component and possibly is derived from it.

Studies of the protein in the ultracentrifuge. In the ultracentrifuge this material appears to be a single homogeneous protein with a molecular weight of about

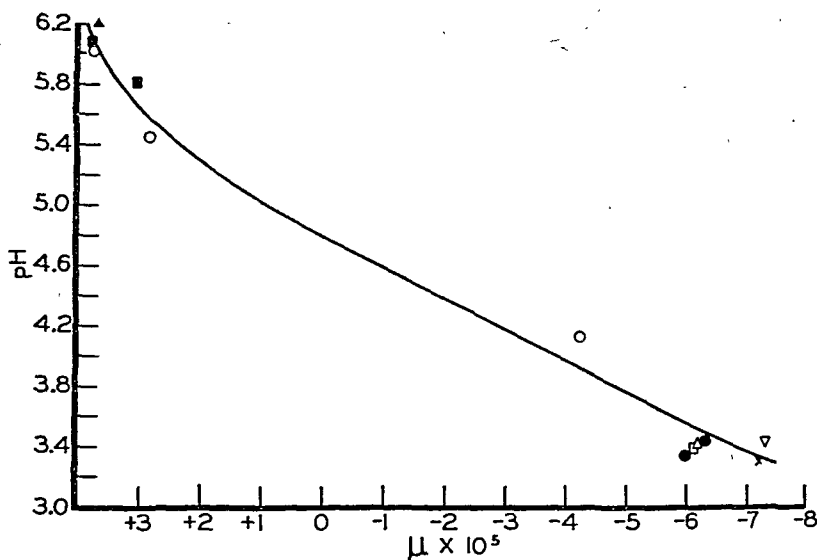


FIG. 3. MOBILITY OF THE PROTEIN IN RELATION TO pH AS DETERMINED IN THE ELECTROPHORESIS APPARATUS OF TISELIUS

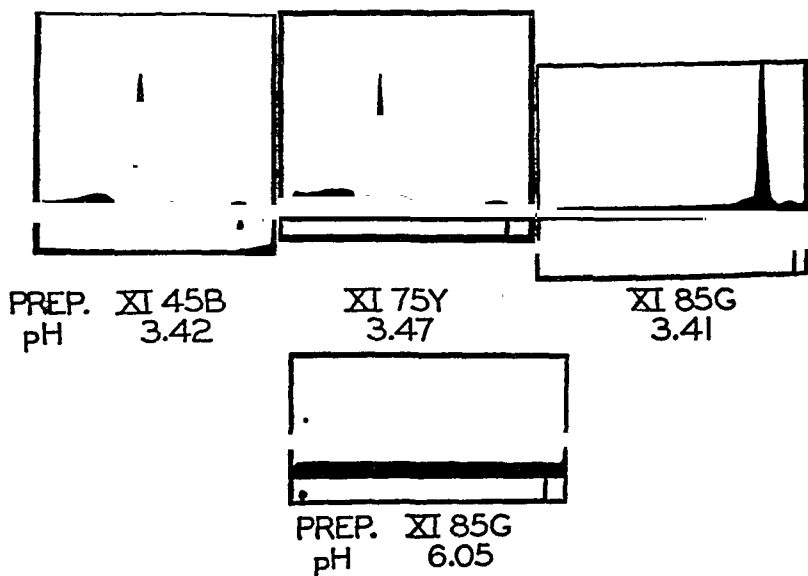


FIG. 4. ELECTROPHORETIC PATTERNS OF THE PROTEIN. SEE TEXT

30,000. The data on which this statement is based are summarized in table 1, which includes a determination of the diffusion coefficient of the first solution. The diffusion constant there given was determined in an apparatus similar to the one used for the electrophoresis experiments in which the Longworth (11)

TABLE I
Ultracentrifugation studies of pure active protein of the pars neuralis of the ox

PROTEIN CONCENTRATION	SOLVENT	δ_{20}	η_{sp} (POISES)	t	S_1 (EXPERIM.)	S_{20}^\dagger	M	REMARKS
per cent				°C.				
1.05	0.1 M acetate buffer, pH 3.30	1.0009	0.01043	7.8	1.98×10^{-13}	2.80×10^{-13}	31,200	Diffusion coefficient determined with same solution, $D_{0.1} = 4.4 \times 10^{-7}$; $D_{2.0}$ (calc.) = 8.5×10^{-7}
0.50	0.34 M NaCl and 0.05 M acetate buffer, pH 3.38	1.013*	0.0103*	22.0	2.59×10^{-13}	2.61×10^{-13}	29,700	Not completely homogeneous
0.77	0.17 M NaCl and 0.02 M acetate buffer, pH 3.64	1.0055	0.01026	17.6	2.37×10^{-13}	2.61×10^{-13}	29,700	
1.10	0.17 M NaCl and 0.05 M acetate buffer, pH 3.35	1.0059	0.01028	18.7	2.42×10^{-13}	2.61×10^{-13}	29,700	Also used for separation expt.
0.19	0.17 M NaCl and 0.1 M acetate buffer, pH 3.35	1.0099	0.01024	18.2	1.6×10^{-13}	2.38×10^{-13}		Inaccurate. Boundaries blurred and gradient too low

* Estimated.

† Calculated constant of sedimentation in water at 20°. 0.749 was assumed to be the specific volume at 20°. $\frac{f}{f_0} = 1.15$.

scanning device was employed to obtain the different patterns. At the beginning of the experiment the boundary was slowly pushed into the middle of the Tiselius cell by a compensating device, and the solution was allowed to diffuse into the acetate buffer against which it had been dialyzed. The constant was determined by the formula

$$D = \frac{S^2}{4\pi l H_{\max}^2}$$

where S is the diffusion area in cm^2 , t the time in seconds, H_{\max} the maximum height of the curve in cm.

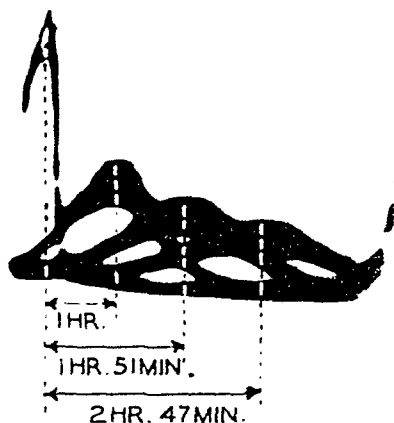


FIG. 5

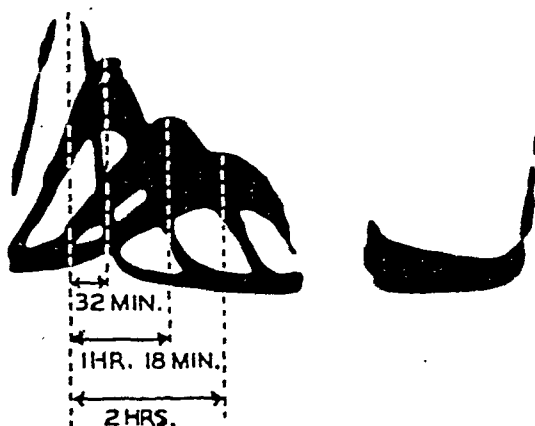


FIG. 6

FIGS. 5 AND 6. SEDIMENTATION PATTERNS OF THE PROTEIN. SEPARATION CELL USED IN OBTAINING PATTERNS OF FIG. 6

Examples of sedimentation patterns are reproduced in figures 5 and 6. Homogeneity is shown by the symmetry of the curves and by the absence of significant displacement of the base line. The specific volume of the protein is assumed to

be 0.749 leading to the calculation of the molecular weight from the usual formula

$$M = \frac{RT_s}{D(1 - V\rho)} \approx 30,000$$

where s is the sedimentation constant, D the diffusion constant, V the specific volume of the protein, ρ the density of the solvent, R the gas constant, and T the absolute temperature. The ratio $\frac{f}{f_0} \approx 1.15$ corresponds to the average value found for most proteins. It means that the molecule can be considered an oblong ellipsoid whose two axes have a maximum ratio of 3.5 provided that there is no solvation.

TABLE 2

Some chemical and physical properties of pure active protein of the posterior lobe of the ox

Elementary analysis:	per cent
Carbon.....	48.64
Hydrogen	6.63
Nitrogen.....	16.32
Amino nitrogen.....	0.054
Phosphorus	0.027
Sulfur.....	4.89
Chlorine	0.02
Ash.....	0.58
Oxygen (by difference)	22.89
Distribution of sulfur:	
Cysteine (nitroprusside test).....	0
Cystine.....	4.3
Methionine	?
Sulfate.....	0.1-0.4
Molecular weight.....	30,000
Isoelectric point.....	4.8-4.9

Other properties of the protein. Elementary analysis of the protein yielded interesting results which are assembled in table 2. If one assumes that there is present only one free amino group per molecule, the calculated molecular weight would be about 26,000,—a figure in fair agreement with that obtained from the sedimentation constant during ultracentrifugation. The low phosphorus content indicates that the protein is not a nucleo-protein. The high percentage of sulfur led to an investigation of the distribution of this element. In Anson's nitro-prusside qualitative test for free sulfhydryl groups (9), the protein is denatured by guanidine. This test was negative for as much as 2 mgm. of the posterior lobe protein but was positive for 0.085 mgm. of egg albumin. It was therefore concluded that the molecule contains no cysteine. Cysteine-cystine, methionine, and sulfate were all determined by the Kassel-Brand modification of Baernstein's method (8). Although samples for analysis were small, the results indicated that methionine probably is not present and that, in view

of the negative results in testing for —SH groups, nearly all the sulfur is present as cystine sulfur.

THE BIOLOGICAL ACTIVITY OF THE PROTEIN. As many methods of biological assay as appeared to have quantitative value were employed. An especially large number of assays was made of preparation XI-153. Therefore, it seemed best to present these results in some detail to give the reader an idea of the variability encountered by different methods. All assays were performed "blindly," i.e., the concentration of active substance was known to the assayist only to the extent that it fell within broad limits. The U.S.P. reference standard was used in all cases.

On the basis of the experiment of table 3, it is believed that about 20 micrograms of N of the protein are equivalent to 1 mgm. of U.S.P. reference standard

TABLE 3
The potency of preparation XI-153 in inhibiting water-diuresis in the rat

SUBSTANCE INJECTED	DOSE		NUMBER OF RATS	T = AVERAGE TIME REQUIRED FOR EXCRETION OF 50 PER CENT OF URINE	RATIO OF POTENCY	
	U.S.P. unit per kgm.	Micrograms N per kgm.			$\frac{TC}{TB}$	$\frac{TC - TA}{TB - TA}$
A' Control.....			32	min. 62.3		
B U.S.P. Stand.....	0.01		32	118.5	0.94	0.88
C Post. lobe protein.....		0.08	32	111.8		
B U.S.P. Stand.....	0.01		32	112.6	1.03	1.07
C Post. lobe protein.....		0.10	32	115.8		
B U.S.P. Stand.....	0.01		32	109.0	1.13	1.31
C Post. lobe protein.....		0.12	32	122.8		
A'' Control.....			32	66.0		

powder in diuresis-inhibiting action. The same group of 32 rats was used in all the experiments. These were divided into subgroups of 4 each after the technique of Burn (19). The rats were all males weighing about 200 grams. Fluid was not administered by stomach tube as Burn recommended but by the intraperitoneal injection of 50 cc. of 0.3 per cent NaCl per kilogram body weight just before the extract was injected subcutaneously. A given comparison, such as that of the standard (0.01 unit per kilogram) with the protein was performed at an interval of 3 or 4 days so that 4 subgroups receives standard or protein each time with reversal of the groups used for a particular comparison. There was considerable variation in the time of inhibition caused by the standard administered at only one dose level. However, the comparison with the protein led to consistent results. Less elaborate comparisons were made with 4 other pure preparations of the protein. In no case was the apparent potency of the protein represented by a value 20 per cent less than the value given in table 4.

Assays of preparation XI-153 by various methods are summarized in table 4. With the exception of the effect on melanosome-dispersion in the frog, the results indicate that the oxytocic (isolated guinea-pig uterus or fowl blood-pressure), pressor (blood-pressure of cat or dog) or diuresis-inhibiting activity of the preparation is represented by about 10 micrograms of N per U.S.P. unit. In our experience assays of pressor activity are often less reproducible than the results with preparation XI-153 indicate. Pressor activity of other preparations of pure protein in some assays was represented by as much as 12.5 micrograms of N equivalent to 1 U.S.P. unit.

Melanosome-dispersion was studied in normal or hypophysectomized frogs with three different pure preparations. The results were consistent and indicated, as shown in table 4, that U.S.P. extract is 2000 times as potent as our *pars neuralis* protein when this activity is tested in terms of oxytocic-vasopressor units. In the particular experiment cited, the solution of U.S.P. extract (after removal of substances insoluble in the dilute acetic acid) and the solution of

TABLE 4

Biological activity of pure active protein of the posterior lobe of the ox (preparation XI-153)
(No significant deviations were encountered in the assay of four other preparations)

METHOD OF ASSAY	NUMBER OF ASSAYS	AVERAGE AND S.E. OF MICROGRAMS PROTEIN NITROGEN EQUIVALENT TO ONE U.S.P. UNIT
Isolated guinea pig uterus.....	11	9.3 \pm 0.5
Blood-pressure of fowl.....	26	10.4 \pm 0.4
Blood-pressure of cat.....	5	10.4 \pm 0.4
Blood-pressure of dog.....	2	9.3
Diuresis-inhibition in rat.....		10.0*
Melanosome-dispersion in frog.....		20,000

* See table 3 and text.

pars neuralis protein were adjusted to pH 10.5, placed in a boiling water bath for 4 minutes, chilled, and acidified to pH 5.0.² In hypophysectomized frogs the threshold dose of alkali-treated filtered acid extract of U.S.P. powder was found to be about 0.00025 unit per frog.

An international (oxytocic) unit of U.S.P. reference powder is represented by the extract of 0.5 mgm. of powder. Authors often fail to emphasize that the final extract contains far less organic material than that probably contained in the original powder. For example, we commonly make standard extract for assay equivalent to 2 mgm. (4 units) U.S.P. powder per ml. The amount of nitrogen per U.S.P. unit in this solution is 20.3 micrograms. The figure may be higher in commercial posterior pituitary extracts (e.g., 35 micrograms N per unit). Commercial samples of Pitocin and Pitressin vary widely in the amount of nitrogen per unit; the lowest figures found by us were 4.6 micrograms of N

² We owe to Dr. E. M. K. Geiling the suggestion that melanosome-dispersing activity could be more convincingly evaluated if the material were first treated with alkali.

for 1 unit of Pitocin and 3.9 micrograms of N for 1 unit of Pitressin determined from solutions of either extract as marketed commercially. Commercial Pitocin was found to contain very little melanosome-dispersing activity (0.2 per cent that of U.S.P. extract per oxytocic unit) whereas Pitressin had perhaps 50 per cent the activity of U.S.P. extract in this respect. Both comparisons were based on oxytocic or pressor units.

EVIDENCE THAT THE BIOLOGICAL ACTIVITIES ARE CHARACTERISTICS OF THE PROTEIN. It is well known that various investigators, notably Dudley, Kamm and his collaborators, and later, Stehle, du Vigneaud and their colleagues, have been able partly or nearly completely to separate the oxytocic and vasopressor activities from each other. Therefore it was of great importance to demonstrate as convincingly as possible whether the two principal activities of posterior lobe extract are or are not part of the pure protein isolated according to the method we have described. Later we shall discuss the reconciliation of our findings with those of workers who isolated the two activities as separate substances of much higher potency.

Within the limitations of careful biological assay, different preparations of the pure protein are identical in either oxytocic or vasopressor activity in which one U.S.P. unit is represented by about 10 micrograms of protein nitrogen or 61 micrograms of protein. This statement is based upon work with five different preparations of the protein made from as many different batches of starting material. Such findings support the belief that oxytocic and vasopressor (including diuresis-inhibiting) activities are part of the protein and are present in ratios resembling those found in cruder extracts of posterior lobes of oxen. To furnish further evidence there will now be described efforts to detect oxytocic or vasopressor activities more potent than those of the pure protein. For this purpose fractions were secured (a) after electrophoretic migration of the protein, (b) after solubility tests, and (c) after ultracentrifugation.

Fractions secured after electrophoretic migration of the protein. After a dissolved protein has been electrolyzed in the Tiselius apparatus migration will occur slowly or rapidly depending mainly upon how close the pH of the solvent is to the isoelectric point. If migration is rapid or if electrolysis is continued long enough, fractions of the solution can be withdrawn from the limbs of the cells containing either virtually no protein or pure protein. In addition, as shown in figure 4, there appears to be a second component at pH 3.4-3.5 which could be separated from the main component and assayed. (At other pH values the same preparations appeared as one component which, however, was electrophoretically inhomogeneous.) The assays of all fractions were undertaken after nitrogen determinations had been made. The concentration of nitrogen in the "protein free" fractions was usually so low (8-20 micrograms per ml.) that the error of determination was relatively high and, in view of the lack of any change in potency, necessitated assay by only very sensitive methods such as the depressor effect on fowl blood-pressure.

Our results can be summarized by stating that after electrolysis of solutions of the protein at pH 3.4-3.5, the protein and "protein-free" fractions were indis-

tinguishable in activity in terms of nitrogen. The minor component appearing at pH 3.4-3.5 likewise did not differ significantly in potency when compared with the main component—a fact suggesting that it is derived from or closely related to the main component.

Fractions secured in solubility tests. In the experiment of figure 7, protein to various concentrations was dissolved in 0.5 M acetate buffer, pH = 3.90, and the equivalent of 6.5 grams of NaCl per 100 ml. was added. After the NaCl had been dissolved, only the first three mixtures (0.048-0.072 mgm. N per ml.) were clear solutions; the protein in excess of saturation was in suspension in the

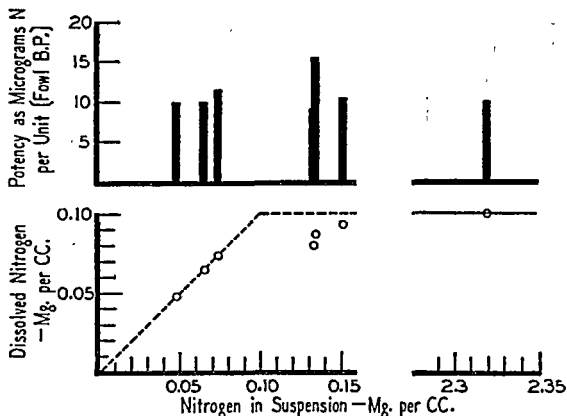


FIG. 7. THE OXYTOMIC ACTIVITY OF DISSOLVED PROTEIN IN RELATION TO AMOUNT DISSOLVED OR SUSPENDED IN 0.5 M ACETATE BUFFER, pH = 3.90, TO WHICH 6.5 GRAMS NaCl PER 100 ML. WERE ADDED

remaining solutions. The oxytomic activity (depressor effect on fowl blood-pressure) was determined in the supernatant fluids (dissolved protein). With the exception of one aberrant result (at about 0.135 mgm. of suspended protein nitrogen) which was not confirmed on immediate repetition, there were no significant variations in the activity of the supernatants. Had a less soluble potent impurity been present, the activity of the supernatant would have been lower when there was a great excess of suspended protein. If a more soluble potent impurity had been a contaminant, the activity would have been lower when all the protein was dissolved or when that present was only moderately in excess of saturation.

The legend of figure 8 describes the conditions under which that experiment

was performed. If a less soluble active impurity (hormone) had been present, the washed precipitated protein, *P*, would have been the more potent, and the supernatant, containing dissolved protein as *S*, would have been the less potent. If a more soluble active impurity (hormone) had been present, *P* would have been the less potent and *S* would have been the more potent. The amount of material available permitted two careful assays of *P* and *S* by both techniques. The mean results which are plotted show that the activities of *P* and *S* did not

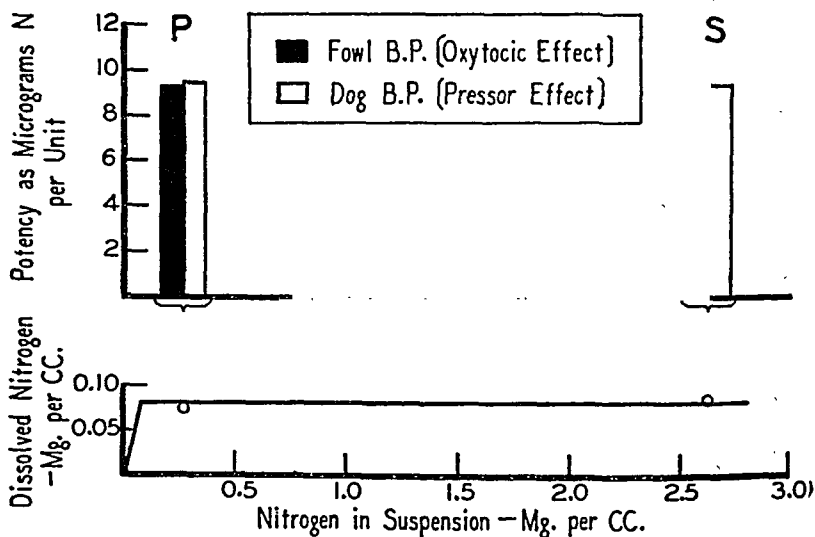


FIG. 8. The lower curve represents the solubility of pars neuralis protein. At the first point (0.266 mgm. N in suspension), the protein used had been washed three times by dissolving in 0.5 *M* acetate buffer (pH 3.95) and precipitating by the addition of NaCl (6.5 grams per 100 cc. of solvent). In this way, 54 per cent of the starting material had been removed. The oxytocic and pressor assays of this protein as the insoluble portion at the point indicated are given under *P* (upper rectangles). Assay of the supernatant (dissolved protein) was performed at *S* from a suspension (made by dissolving the protein in 0.5 *M* acetate buffer and adding 6.5 grams NaCl per 100 cc. of solvent) ten times as concentrated as at *P* and thirty times the concentration of protein in saturated solution in the NaCl acetate solvent.

The pharmacological activities of *P* and *S* whether tested for oxytocic or pressor effects do not differ significantly.

differ significantly in respect of either oxytocic or pressor effects. Therefore this experiment likewise supports the beliefs that oxytocic and pressor activities in a ratio similar to that of U.S.P. reference standard are parts of the pure protein and that principles more active than the protein could not be detected.

Fractions secured after ultracentrifugation of a solution of the protein. In experiments of this type the ultracentrifugation cell had a capacity of only 0.40 cc. Therefore assays were based, not upon the nitrogen of a given fraction of the column of liquid, but upon the distribution and total number of units placed in the cell before ultracentrifugation. Control assays of the same protein solution

not subjected to ultracentrifugation were performed for all the comparisons. In all cases solutions of the pure protein of the usual potency (10 micrograms $N \approx 1$ U.S.P. reference standard unit) were employed.

In the experiment of figure 9, the protein was found to be pure and was centrifuged until nearly all appeared to be contained in the lower half of the cell. Assay of the three fractions of the column showed that very little of the total activity (about 5 per cent) could be found in the upper half of the cell. About 6 per cent of the theoretical total activity could not be accounted for. It appears

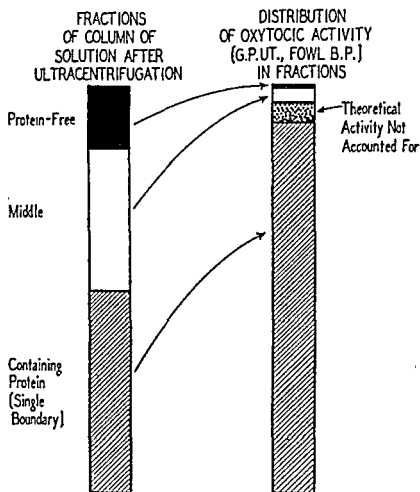


FIG. 9. ACTIVITY OF COLUMNS OF LIQUID IN CELL AFTER ULTRACENTRIFUGATION OF PROTEIN AS 1.05 PER CENT SOLUTION IN 0.1 M ACETATE BUFFER, pH = 3.30

fair to conclude that backward diffusion accounts for the small amount of active material in the protein-free portions of the column of liquid and that activity is associated with the protein.

It will be noted that assays for pressor activity were not performed in the experiment of figure 9. In the separation experiment of figure 10 (see also table 1 and fig. 6), both oxytocic and pressor activities were assayed in the liquid of the two parts of a separation cell³ after the protein, sedimenting as a single

³ The sieve plate supporting a piece of hardened filter paper was located at about a third of the height of the cell from the bottom. The total volume of the cell was 0.9 ml.

component, had all been collected in the lowest third of the cell. The slight activity found in the upper two thirds of the cell corresponded to the amount of protein left as estimated optically. A complete separation was impossible since diffusion, owing to the low molecular weight of the protein, caused an appreciable spreading of the boundary. Rectangles I, II, and III show how oxytocic and pressor activities were distributed in the protein-free and protein-containing parts of the cell. The theoretical total activity was computed from assays performed simultaneously on the same protein solution which had not undergone ultracentrifugation. It is clear that practically all the activity, whether oxytocic or pressor, is associated with the pure protein and that again there is no evidence favoring the presence of additional small non-protein components with high biological activity.

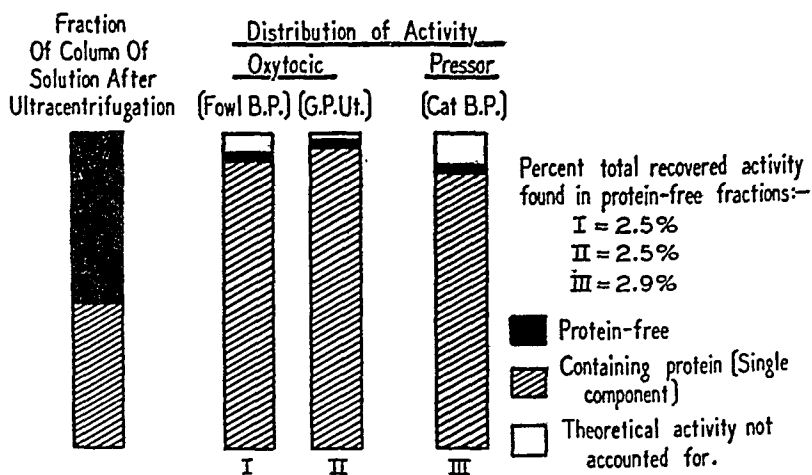


FIG. 10. Experiment similar to fig. 9. See also fig. 6. Ultracentrifugation of 1.10 per cent solution of protein in 0.17 *M* NaCl containing 0.05 *M* acetate buffer, pH = 3.35.

MISCELLANEOUS OBSERVATIONS. *Digestion by proteolytic enzymes.* A solution of the protein at pH 3 was digested by crystalline pepsin in an incubator (37°) for six days so that 36 per cent of the protein present had been digested as determined by the amount of non-protein N appearing. The incubated control had the same activity as the control kept in the refrigerator; the digested protein had about 75 per cent of the activity of the controls. Digestion by crystalline trypsin (70 per cent of protein digested) or crystalline chymotrypsin (95 per cent of protein digested) at pH 7.6 for 43 hours caused virtually complete destruction of activity compared with the control solution incubated at the same pH. In all these experiments only oxytocic activity was tested. The results are about what would be expected in view of earlier work on posterior lobe extracts.

The effect of reduction of —S—S— groups. Since in our protein nearly all the sulfur appears to be in the form of cystine, we made a preliminary investiga-

tion of the activity after reduction of the protein by thioglycollic acid or cysteine. Presumably dithio groups of cystine would thus be reduced to sulphydryl groups.

In a typical experiment, 2.0 ml. of posterior lobe protein (0.364 mgm. N per ml.) were mixed with an equal volume of saline or of neutralized thioglycollic acid and 0.4 ml. of 0.1 N NaOH (final pH ca 7.5). Equal volumes of saline and thioglycollate were mixed to serve as another control. The protein solution not containing thioglycollic acid could be assayed only after it had been greatly diluted. The control solution containing only thioglycollic acid as well as the protein solution which had undergone reduction were injected in equally large doses of diluted mixture into the dog or fowl used for assay. Reduction of the protein by thioglycollate under the described conditions abolished more than 99.5 per cent of the activity when reduction had been allowed to proceed no longer than 2-5 minutes (5 experiments). Both vasopressor and oxytocic activities were affected to an equal extent. The alkalinized solutions of protein (non-reduced controls) appeared to be as active as saline solutions of the protein. The thioglycollic acid control solutions produced no pharmacological effect.

When similar experiments were performed with cysteine, the amino acid had a less pronounced effect probably because it is a less powerful reducing agent than thioglycollate. The pH of the solutions, whether controls or containing 30 mgm. of cysteine HCl for 0.182 mgm. of protein N, was raised to about 7.5. Cysteine Na solutions had no effect in doses equalling the maximum used for other solutions. After 2 minutes' reduction, the maximum loss of pressor activity was 44 per cent (2 experiments). After 5 minutes' reduction (2 experiments) 76 and 85 per cent of the oxytocic activity had disappeared. (The difference in time of reduction was arbitrarily chosen and has no significance.)

The action of the protein on the concentration of blood sugar. Doses equivalent to 1 and 3 U.S.P. units per kilo injected subcutaneously into fasted rabbits had no effect on the level of blood sugar estimated by the method of Shaffer and Hartmann as modified by Somogyi (22). Duplicate samples of blood were withdrawn before injection as well as at 1, 1.5, 2, and 3 hours after injection. Five units of the protein per kilogram body weight caused no change in the blood-sugar level of another rabbit. The subcutaneous administration of 2 units per kilogram of commercial posterior pituitary extract caused a moderate elevation of the blood glucose (77 mgm. per cent before injection; maximum rise to 119 mgm. per cent 1.5 hours after injection). We concluded that the protein probably does not affect carbohydrate metabolism so far as this can be judged by acute changes in the level of blood sugar.

DISCUSSION. The protein which we extracted from freshly frozen posterior lobes of oxen appears to contain, in constant amounts, multiple biological activities as shown by its ability to cause uterine contraction, a depressor effect in the fowl, a pressor effect in the cat and dog, and inhibition of diuresis in the rat.⁴ These activities are present in about the same ratios as in expressed

⁴ MacArthur (23) has not reported *in extenso* on the nature of the substance with multiple activities which he isolated.

tissue fluid or simple acid extracts of posterior lobes of oxen. Our results therefore harmonize well with Rosenfeld's study (7) of the ultracentrifugal sedimentation of oxytocic and pressor activity. This author suggested that the two activities can be separated by cleavage from a parent molecule and then exist as much smaller molecules sedimenting at a much slower rate. However if the protein-containing extract is kept as native as possible by avoiding heating in acid solution or some other drastic procedure, then the two activities sediment as if part of one (or more) protein molecules. The late Professor Abel long championed a similar view which he maintained in his last publication in this field (2). However, as Dale emphasized, the presence of large amounts of the melanosome-dispersing ("melanophore") hormone in his preparations is a valid argument against his position since this hormone is derived not from the *pars neuralis* but from the *pars intermedia*. The protein described in this report contains virtually no intermedin.

Many investigators have commented on the constancy of the ratio of oxytocic and vasopressor activities of beef posterior lobes and this fact suggests that these activities may be part of one molecule. However, in the whale it has been reported (24, 25) that oxytocic activity may be 8-10 per cent (sperm whale), 30 per cent (blue whale), or 40 per cent (finback) of the vasopressor activity in terms of U.S.P. reference standard. Although such findings can obviously be interpreted in different ways until more data have been gathered, they speak against rather than in favor of the belief that both activities are part of one molecule.

As a result of the pioneer work of Dudley (3) and especially of Kamm and his colleagues (4) followed by the confirmatory experiments of Stehle, du Vigneaud and their collaborators there can be no doubt that oxytocic and vasopressor principles can be separated from each other in highly potent form. The most potent oxytocic preparation reported contained 1 unit in 2 micrograms of solids (30 times the potency of the protein reported here); a vasopressor preparation containing 1 unit in 5 micrograms of solids (12 times as potent as our protein) has been described. There appear to be two possible explanations permitting reconciliation of these facts with our belief that a protein with multiple activities also can be isolated: (1) the protein isolated, although pure to the extent that present physico-chemical methods permit such a conclusion, is pharmacologically active because of the adsorption of the highly active separated principles, or (2) the protein, in part composed of active principles which can be separated from it, is elaborated by and stored in the *pars neuralis*.

It is well known that differential adsorption of the vasopressor principle by bentonite or artificial zeolites occurs easily under proper conditions (26, 27). The fact that this can be effected on an artificial zeolite if a posterior lobe extract in which cleavage of protein has been avoided be used (27), does not demand the conclusion that vasopressor activity is only in loose physical union with the proteins of the gland. A powerful adsorbent can be considered to affect drastically molecules as fragile as protein molecules. Oxytocic activity can be adsorbed by Fuller's earth, norite, talcum and PbS. Also, apparently pure crys-

talline hormone with both activities present, has been isolated on more than one occasion only to lose its biological activity with repeated recrystallization (28). In these instances we appear to be dealing with adsorption of the active principles and it cannot be denied that the biological activity of the protein here described may depend upon adsorbed oxytocic and vasopressor principles rather than upon chemical union (e.g. peptide linkages) of these principles with the protein. However, all the evidence we have obtained—constancy of activity in different preparations, absence of potency greater than that of the protein in fractions secured after electrophoresis, ultracentrifugation, or solubility tests, association of activity with the protein,—supports the view that the activities are part of the protein.

Moreover, the following experimental facts are explained only with great difficulty by the adsorption theory. It was found⁵ that the protein carrying the oxytocic-pressor activity could be spread as a surface film in an unfolded condition, since the thickness of the film was only 8 Å as measured by the optical method of Blodgett and Langmuir (29). If the activities had consisted of small units adsorbed to the protein, either the adsorbed active units would have gone into solution when the unfolding occurred or they would have continued to be associated with the denatured protein of the film. In the first case the underlying fluid should have contained all the activities and in the second the activities should have been recovered in the film, since it was not to be expected that unfolding of the hypothetical adsorbing protein should affect the potency of the assumed small units. The film was found to be nearly completely inactive and the potency of the underlying fluid in terms of protein N was less than that of the original material similarly diluted and concentrated for purposes of control. (The activity present could be accounted for by the amount of protein going into solution at the time of spreading.) It can be concluded that unfolding virtually destroys the activities indicating that some part or parts of the original configuration must remain intact if potency is to be preserved.

If the protein were subjected to heat perhaps at pH 3.5–4.0 following which the dialyzability of the two (or more) principles were to be investigated, useful information might be secured from following the ratio of activities dialyzed or retained (28). In our opinion, this information can be obtained only from careful serial experiments in which the rate of change is followed. We hope later to perform such experiments.

The second possible reconciling explanation is that a protein containing all the activities is formed in the *pars neuralis* but that drastic methods of extraction, autolysis, etc. facilitate or cause the liberation or cleavage of the active principles. An important and inescapable question is: If only a single protein with all activities is elaborated in the *pars neuralis*, is it secreted unchanged? Teleologically at least this appears to be improbable and should not be accepted without proof (28). Possibly specific enzymes liberate one or the other active fragments of the parent molecule depending upon specific demands of the

⁵ Cold Spring Harbor Symposia, 9: 272, 1941.

organism. So far as we are aware there have been no careful quantitative assays in search of multiple activities in single specimens of urine or blood under conditions leading to the expectation that at least one activity (e.g., that inhibiting diuresis) would be secreted in increased amounts. Such a problem is exceptionally difficult to solve and would demand elaborate preliminary control experiments.

We have already referred to the fact that the dialysate of crude extracts obtained from fractionating extracts of fresh glands contains a considerable amount of biologically active material. It is our belief that this is probably the oxytocic and vasopressor principles, whatever their origin, mixed with other non-protein nitrogenous substances. Large quantities of perfectly fresh posterior lobes of oxen cannot ordinarily be obtained but must be collected, frozen and later dissected. If the protein is the sole source of active principles possibly it undergoes *post mortem* enzymatic cleavage sufficiently to account for any coexisting dialyzable principles which are presumed to be separate.

The cystine content of the protein we isolated is extraordinarily high (16.1 per cent or 4.3 per cent cystine sulfur). It appears that all the sulfur is present as this amino acid. Other investigators have reported the sulfur content of purified oxytocic or vasopressor principles as ranging from 3.1–4.5 per cent. Improved methods of determining cystine-cysteine and methionine have become available since these reports were made and the figures given by earlier authors as representing cystine cannot justifiably be compared with ours. However, it appeared that about 10–12 per cent of highly purified oxytocic or vasopressor principle was represented by cystine. It is noteworthy that in purifying the vasopressor principle Irving, Dyer and du Vigneaud (30) early separated an impurity containing a large amount of cystine. Although Sealock and du Vigneaud (31) found that whether the sulfur was present in $-S-S-$ or $-SH$ form (reduced by cysteine) seemed not to affect either vasopressor or oxytocic activity, Gulland and Randall (32) as well as Freudenberg and his collaborators (33) concluded that reduction definitely impairs activity at least of the oxytocic principle. Freudenberg and others found that nearly complete inactivation followed treatment of purified oxytocin with neutral or weakly alkaline sulfite or with hydrogen in the presence of Pd; sulfite as well as other means of reduction were employed by Gulland and Randall. In our own experiments, reduction by cysteine lowered both vasopressor and oxytocic (fowl depressor) potency of solutions of the protein. Reduction by thioglycollic acid caused nearly complete inactivation. Therefore we believe that the protein becomes inactive if the dithio groups (cystine) are completely reduced to sulphydryl groups.

SUMMARY

The isolation of a protein from frozen posterior lobes of oxen is described. It appears to be pure so far as this can be demonstrated by constant solubility and by "schlieren" patterns in the ultracentrifuge. Although some inhomogeneity was found in the Tiselius electrophoresis apparatus, reasons are given for believing this to be derived from a pure protein. The molecular weight is

about 30,000. The isoelectric point is about pH 4.8. Empirical analysis revealed an unusually high percentage of S (4.9 per cent) which is almost entirely present as part of cystine.

Oxytocic, vasopressor, and diuresis-inhibiting activities are all present in ratios resembling those of U.S.P. reference standard. Ten micrograms of nitrogen or 61 micrograms of solids are approximately equivalent to 1 U.S.P. unit. Experiments designed to demonstrate that the oxytocic and vasopressor activities are moieties chemically united with the protein are described.

Reduction of the cystine in the protein by thioglycolic acid nearly abolishes the activity. In this respect cysteine is much less effective.

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THE EFFECT OF MULTIPLE SUBSTITUENTS ON THE TOXICITY AND TREPONEMICIDAL ACTIVITY OF PHENYLARSENOXIDE

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It has been shown in preceding papers of this series (1, 2, 3) that simple substituent groups introduced into phenylarsenoxide might have any one of three effects on its toxicity and treponemicidal activity. As is indicated in table 1, a large group of substituents had either no significant effect, or depressed the activity and toxicity to approximately the same degree, resulting in compounds with essentially the same (unfavorable) "chemotherapeutic index" as that of phenylarsenoxide (table 1, section A). When an acidic group ($-\text{SO}_3\text{H}$, $-\text{COOH}$, $-\text{RCOOH}$) was introduced into the phenyl ring, it regularly caused an extraordinary decrease in the treponemicidal activity of phenylarsenoxide without a commensurate decrease in toxicity. In consequence, such compounds had an even more unfavorable ratio of activity:toxicity than did the parent phenylarsenoxide (table 1, section B). However, when the acidic function was blocked, as by ester or amide formation, compounds were obtained in which the ratio of treponemicidal activity:toxicity was not merely restored to that of phenylarsenoxide, but exceeded it significantly (2, 3).¹ Thus, most of the amides had "indices" 3 to 5 times more favorable than that of phenylarsenoxide, due primarily to their low toxicity. A similar, if less pronounced, favorable effect was produced by substitution with acetamido groups, the ratio of activity:toxicity in this series being 2.1 to 2.9 times as favorable as that of phenylarsenoxide (1). A barely significant favorable effect was produced by $-\text{NH}_2$ and $-\text{OH}$ substitution in the *meta* or *para* position (1) (compounds of each type are illustrated in Table 1, section C).

Given the widely varying effects of individual substituent groups on the toxicity and treponemicidal activity of phenylarsenoxide, the compounds described in the present paper were prepared in order to determine their effects when used in combination.

METHODS AND MATERIALS. The methods of assaying treponemicidal activity and toxicity have been described in the first paper of this series (5).

It is to be noted that in the arsenoxides so far tested, the treponemicidal activity *in vitro*

¹ It is interesting to note that the trypanocidal action of acid-substituted arsonic acids is similarly enhanced by ester or amide formation (4).

has roughly paralleled the therapeutic action *in vivo*, and the relative toxicity in white mice has paralleled the relative toxicity in rabbits. The ratio of treponemicidal activity:mouse toxicity has therefore been a valuable first approximation of the true chemotherapeutic index as observed in syphilitic rabbits.

Of the 27 disubstituted compounds included in the present report, 21 are new compounds. The preparation of 19 of these has been described in detail elsewhere (6). The methods used for the preparation of the remaining compounds are given in the footnotes to table 2. Four compounds (the 3,4-dicarbamyl, 3,5-dicarbamyl, 3,4-dicarbomethoxy, and the 3-acet-amido-4-carbamyl phenylarsenoxides) are not included because they were hydrolyzed in dilute alkali to a significant degree. A fifth compound, the 3-amino-4-acetoxy phenylarsenoxide, could not be included because its low solubility at pH 7.0 precluded the testing of biological activity.

TABLE 1

The effect of some single substituents on the treponemicidal activity in vitro and toxicity of phenylarsenoxide

The following tabulation includes only those groups represented in the di- and tri-substituted compounds of table 2. The values for the treponemicidal activity and the toxicity per mole are referred to those of phenylarsenoxide as 100, and the activity:toxicity ratio is referred to that of phenylarsenoxide as 1.

A NO SIGNIFICANT EFFECT ON ACTIVITY:TOXICITY RATIO (cf. (1))				B UNFAVORABLE EFFECT, I. E., ACTIVITY:TOXICITY DECREASED (cf. (2))				C FAVORABLE EFFECT, I. E., ACTIVITY:TOXICITY RATIO INCREASED (cf. (1, 2, 3))			
Compound	Relative treponemi- cidal activity*	Relative toxicity*	Activity:toxicity ratio	Compound	Relative treponemi- cidal activity*	Relative toxicity*	Activity:toxicity ratio	Compound	Relative treponemi- cidal activity*	Relative toxicity*	Activity:toxicity ratio
2-CH ₃	83	84	1.03	4-COONa·H ₂ O	6.7	41.1	0.16	3-NH ₂	104	79.6	1.31
3-NO ₂	89	80	1.11	4-SO ₃ Na 3H ₂ O	3.4	29.0	0.12	4-NH ₂ 2H ₂ O	83	66.6	1.46
3-Cl	83	77	1.08					3(3)-OH 2H ₂ O	78.5	49	1.61
3-Cl	110	110	1.00					4-OH H ₂ O	72	49	1.47
4-Cl	85	99	0.86					4-C ₂ H ₅ OH	78.5	51	1.62
4-OCH ₃	127	118	1.03					3-NHCOCH ₃ ·H ₂ O	41.5	19	2.18
2-OH	84	86	0.99					4-NHCOCH ₃	56.7	20.5	2.76
2-NH ₂	83	84	1.04					4-CONH ₂	44.5	9.6	4.6

* Per mole, i. e., per unit As, and referred to that of the unsubstituted phenylarsenoxide as 100 (cf. (1-4)).

EXPERIMENTAL RESULTS. The treponemicidal activities *in vitro*, relative to that of phenylarsenoxide as 100, are given in column 2 of table 2. Each value in that column is the mean of 4 to 8 determinations. As is evident from the table, the standard error of a series of determinations on the same compound averaged less than 10 per cent.

The toxicity in mice, expressed as the amount per kilogram which killed 50 per cent of the animals in four days, is given in columns 3 and 4 of table 2. In column 5, the molar toxicity is expressed relative to that of phenylarsenoxide as 100.

Finally, the ratios of treponemicidal activity:toxicity, which may be taken as a first approximation of potential therapeutic utility, are given in the last column of table 2.

ity:toxicity ratio of phenylarsenoxide, did not improve when used in combination, while the favorable effect on an amino, hydroxy, or amide substituent was usually masked by a second inert or unfavorable group.

The foregoing results were perhaps to have been anticipated. What was not anticipated was the effect of multiple substituents, when each individual substituent was known to have a favorable effect on the activity:toxicity ratio. Fifteen such compounds (numbers 13 to 27) are listed in table 2.

In most of these combinations (cf. compounds 13-17, 22, 23, 25, 26 of table 2) there was no evidence of an additive effect. The activity:toxicity ratios of the multiple-substituted compounds were usually no greater, and often distinctly less than the ratio given by the better of the single substituents (cf. 3-OH-5-NH₂ and 3-OH-4-CONH₂). This inhibition of the favorable effect of individual groups when used in combination was also observed in the case of two compounds (numbers 14 and 25) doubly-substituted with the same group.

There was, however, one combination of two favorable groups which did prove to be not merely additive, but actively synergistic. This was the 3-NH₂-4-OH grouping of compound number 18 of table 2 ("Mapharsen"). The toxicity of this compound was strikingly reduced, down to $\frac{1}{4}$ that of phenylarsenoxide, while the treponemicidal activity was decreased by approximately two-thirds. The resulting activity:toxicity ratio of 5.5 for this compound is to be compared with the index of 1.31 for the 3-NH₂ phenylarsenoxide and that of 1.47 for the 4-OH derivative. We are as yet unable to explain the detoxifying effect of the 3,4-aminophenol grouping as present in this compound, and the puzzle it presents is further accentuated by the data obtained with the following nine compounds listed in table 2 (numbers 19-27).

"Mapharsen" is one of ten possible isomeric aminophenols, of which we have prepared and studied six (compounds 18-23). Their treponemicidal activities per mole were quite uniform, varying only between 34 and 57 per cent of that of phenylarsenoxide. However, their toxicity varied to an extraordinary degree (from 7 up to 79 per cent that of the unsubstituted compound) and in consequence, the activity:toxicity ratio, which is a rough measure of potential therapeutic utility, varied from 5.5 to 0.54. In three of the six isomers (compounds number 18, 19, and 20), and unlike any other combination of favorable groups yet studied, —NH₂ and —OH substituents acting in conjunction therefore had a synergistic effect in lowering toxicity, giving compounds with activity:toxicity ratios two to four times more favorable than that given by either group alone. Among those three isomers, the 3-NH₂-4-OH compound stands alone with an index of 5.5. Merely reversing the two groups, as in the 3-OH-4-NH₂ compound, caused a marked increase in toxicity, and a corresponding drop in the activity:toxicity ratio from 5.5 down to 3.9. Of the three other aminophenol isomers listed in table 2 (21, 22, 23) one, the 2-OH-5-NH₂ phenylarsenoxide, had an activity:toxicity ratio of 1.81, only slightly greater than that of the simple *m*-NH₂ derivative, while in the remaining two compounds (3-OH-5-NH₂ and 2-OH-3-NH₂), the toxicity was high, the slight favorable effect of the —NH₂ and —OH groups had been altogether obliterated, and the ratio

of activity:toxicity was even less than that of the unsubstituted phenylarsenoxide (0.78 and 0.54 respectively).

The puzzle presented by the widely varying toxicity and potential therapeutic utility of these six aminophenol isomers, despite their essentially similar treponemicidal activity, finds a counterpart in the difference between the 3,4-diamino and the 3,4-dihydroxy phenylarsenoxides. The monosubstituted —NH_2 and —OH compounds had comparable toxicities and biological activities (cf. table 1). Despite that similarity, the diamino phenylarsenoxide (number 24 of table 2) with a relative toxicity of 15.1, a treponemicidal activity of 53, and an index of 3.5, clearly belongs with "Mapharsen" and its more favorable, non-toxic isomers, while the analogous dihydroxy compound (number 25 of table 2), with a toxicity of 112, and an activity:toxicity ratio of 0.59, is one of the worst compounds in the entire series.

Another unexpected finding was the effect of acetylation on the activity:toxicity ratio of "Mapharsen." As is seen in table 1, acetylation has regularly had a favorable effect on the activity:toxicity ratio of amine-substituted phenylarsenoxides. One would therefore reasonably expect the acetylation of the 3- NH_2 group of "Mapharsen" further to increase its already favorable index. Quite the contrary proved to be the case. The 3- NHCOCH_3 -4-OH compound (compound 13 of table 2) had an index of 1.93, only a fraction as great as that of "Mapharsen" (5.5), and even less favorable than that of the simple 3- NHCOCH_3 compound.² Similarly, the favorable index of "Mapharsen" was largely abolished by the methylation of the amino group (compound 9). The striking synergistic effect of the NH_2 and OH groups as present in "Mapharsen" in decreasing toxicity therefore seems to depend on the integrity of the aminophenol grouping, and has been impaired by any substitution we have yet attempted.

Finally, not only does the integrity of the 3- NH_2 -4-OH grouping seem to be essential for minimal toxicity, and thus for maximal therapeutic utility, but the introduction of a third substituent into the ring has caused a marked decrease in treponemicidal activity and in the activity:toxicity ratio. Two such compounds have been prepared, one with an —NH_2 and one with an —NHCOCH_3 group introduced into the 5- position (compounds number 26 and 27). In both cases the index was decreased, to 2.7 and 1.2 respectively, due entirely to a striking decrease in treponemicidal activity. The unfavorable effect of the 5- NH_2 substitution is particularly surprising, since that compound may be viewed as containing two aminophenol groupings similar to that of "Mapharsen".

SUMMARY

The effect of multiple substituent groups on the potential therapeutic utility (ratio of treponemicidal activity *in vitro*: mouse toxicity) of phenylarsenoxide could not be regularly anticipated from the effect of each of the groups acting singly.

² This observation may be of significance in relation to the utility of "Stovarsol" (3- NHCOCH_3 -4-OH phenylarsonic acid) in the treatment of syphilis.

In general, however, and except for certain of the aminophenols, combinations of 2 or 3 groups were no more favorable, and often were distinctly less favorable, than the best one of the constituent groups:

1) Groups without any significant effect on the ratio of activity:toxicity (2-CH_3 , 3-NH_2 , 2-Cl , 4-OCH_3), when used in combination either remained inert, or lowered the activity:toxicity ratio.

2) Combinations of one "good" group (3- or 4-NH_2 , 3- or 4-OH), and one "inert" or "favorable" group (3- or 4-Cl , 4-OCH_3 , 3-NO_2 , 4-COOH , 2-NH_2) were, with only one exception, not as good as the one favorable group alone, and were often even worse than the unsubstituted parent phenylarsenoxide (cf. the $3\text{-NO}_2\text{-4-OH}$, 3-Cl-4-OH , 2-OH-3-NH_2 compounds).

3) Combinations of two or three "good" groups (3- or 4-NH_2 , 3- or 4-OH , $4\text{-C}_2\text{H}_4\text{OH}$, 3- or 4-NHCOCH_3 , 4-CONH_2) were usually no more favorable than the best of the component groups (cf. $3\text{-NH}_2\text{-4-C}_2\text{H}_4\text{OH}$, $3\text{-NHCOCH}_3\text{-4-OH}$, $3\text{-NH}_2\text{-4-CONH}_2$), and in several instances were distinctly less so (cf. $3,4\text{-diNHCOCH}_3$, $3,4\text{-diOH}$, 3-OH-4-CONH_2 , 3-OH-5-NH_2 , $3\text{-NH}_2\text{-4-OH-5-NHCOCH}_3$).

One combination of favorable groups has, however, proved actively synergistic, causing a striking decrease in toxicity, with only a slight decrease in treponemicidal activity. That favorable combination is the aminophenol group. Six of the ten possible isomeric phenylarsenoxides containing one amino and one phenolic group have been studied here, and three of these ($3\text{-NH}_2\text{-4-OH}$ ("Map-harsen") 3-OH-4-NH_2 , $2\text{-NH}_2\text{-3-OH}$) gave activity:toxicity ratios of 5.5, 3.9, and 3.3 respectively, referred to that of phenylarsenoxide as 1, and to be compared with a maximum index of 1.6 for the single substituent groups. No such synergistic effect was observed in the 2-OH-5-NH_2 , 3-OH-5-NH_2 or 2-OH-3-NH_2 phenylarsenoxides, with indices of 1.8, 0.8 and 0.54 respectively. The favorable index of the $3,4\text{-diNH}_2$ phenylarsenoxide (3.5) may be related to the fact that it could be converted to the corresponding aminophenol by partial oxidation *in vivo*.

The favorable effect of the $3\text{-NH}_2\text{-4-OH}$ combination was impaired by introducing a 5-NH_2 group as a third substituent in the ring, and was altogether obliterated by acetylation of the amino group, methylation of the hydroxyl group, or by introducing 5-NHCOCH_3 as a third substituent in the ring.

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THE EFFECTS OF SOME SPASMOLYTIC SUBSTANCES ON GASTRIC FUNCTION¹

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In a previous paper (1), we examined the spasmolytic actions of some substances related to atropine and "Trasentin." One of these, diethylaminoethyl fluorene-9-carboxylate, appeared to be particularly effective and a promising compound for clinical trial. Since atropine is used for its depressant action on gastric motility and secretion, and since these properties might be undesirable when such a compound is used for other purposes, it seemed indicated to study the modification of gastric activity by this new substance and its congeners.

The cardia. In dogs anesthetized with barbital, the resistance of the cardia to inflowing water was measured by a method like that of Langley (2), but modified for continuous recording. Cardiospasm was produced by bilateral vagotomy. Against such spasm, atropine and substances of the aminoalkyl ester type have a weak and uncertain relaxing action. Papaverine is only occasionally relaxing, and nitroglycerine, which was the most effective of all substances tried, does not always relax this spasm.

Gastric motility. The gastric motility of trained unanesthetized dogs was recorded by a rubber balloon in the stomach, connected with a bromoform manometer. Increased motility was produced by the intravenous injection of insulin (0.2-1 unit per kgm., usually 0.5 unit per kgm.), after a period of starvation of twenty to thirty hours. The spasmolytic substances were injected intravenously. Twenty-four experiments in six dogs were done, and the results are summarized in table 1. Some typical records are shown in figure 1.

Some of these compounds were chosen chiefly to compare their relative relaxing properties on smooth muscle of an organ contracting naturally in the whole animal with their spasmolytic properties as observed by their antagonism to acetyl choline, histamine, and barium ions on isolated strips of intestine. Thus, while nos. 1 and 5 are equally active against histamine and barium, against acetyl choline no. 1 is eleven times more active. With all such comparisons, it is seen that the relaxing potency against this kind of gastric hypermotility is closely related to the potency against acetyl choline, and not to the potency against histamine and barium ions.

The gastric motility in man was similarly studied in three subjects receiving twenty to thirty units of insulin after an eighteen hour fast. Only atropine,

¹ This investigation was aided by a grant from G. D. Searle & Co., Chicago, Ill.

"Syntropan," and diethylaminoethyl fluorene-9-carboxylate were studied. In the record seen in figure 2, it is shown that the fluorene derivative is somewhat more than one-twentieth as active as atropine, while "Syntropan" had no definite effect. These results are quite comparable with those in the dogs. Soreness at the site of injection was noted with the fluorene derivative, not with the other substances.

Gastric emptying time. In four of the trained dogs used in the study of motility, initial and final emptying times of the stomach were observed fluoroscopically, after a meal of 8 gm./kgm. "Junior Milk Bone Dog Biscuit" soaked in 15 cc./kgm. milk, and 2 gm./kgm. barium sulfate. The drugs were injected intramuscularly five minutes before the meal was given. Three control experi-

TABLE 1

NO.	COMPOUND	SPASMOLYTIC ACTIVITY			
		*On small intestine <i>in vitro</i> against			On stomach
		Acetyl choline	Hista- mine	Barium ++	
1	Diethylaminoethyl fluorene-9-carboxylate	1	1	1	
2	Trasentin	6	1.5	1.5	< #1
3	Syntropan	15	30	20	< #1
4	Atropine	0.14	4	20	20 × #1
5	Diethylaminoethyl fluorene-9-acetate	11	1	1	1/10 × #1
6	Diethylaminoethyl 9,10-dihydroanthracene-10-carboxylate	5	0.05	1	< #1
7	Diethylaminoethyl -1phenyl,2-hydroxypropionate	1	20	20	> #3
8	Diethylaminoethyl 1,1-diphenyl,2-hydroxypropionate	5	2	2	< #7
9	Diethylaminoethyl diphenyl chloroacetate	0.7	0.7	1.5	> #1
10	Diethylaminoethyl benzilate	0.7	0.7	2	2 × #1
11	Papaverine	30	1.5	1.5	< #1

* Reciprocal activity ratios from Lehmann and Knoefel (1).

ments, three experiments with a dose of 0.02 mgm./kgm. (0.05 per cent solution) of atropine sulfate, and three experiments with a dose of 2 mgm./kgm. (5 per cent solution) of diethylaminoethyl fluorene-9-carboxylate hydrochloride, were done in each dog. Table 2 gives the average values for each dog and the average for the group.

Gastric Secretion. This was studied in the same four dogs. Five grams of "Armour's Meat Extract" were dissolved in 100 cc. of water and given by stomach tube. Every fifteen minutes a few cubic centimeters of gastric juice were withdrawn until the stomach was empty. Sahli's reagent (equal parts of KI (48 per cent) and KIO₃ (8 per cent)) was used for determination of the free acid. The total acid was determined by titration with N/100 sodium hydroxide with phenolphthalein as indicator. Three control experiments, three experiments after the intramuscular injection of diethylaminoethyl fluorene-9-carbox-

ylate hydrochloride (2 mgm./kgm., 5 per cent solution), and three experiments after the intramuscular injection of atropine sulfate (0.02 mgm./kgm., 0.05

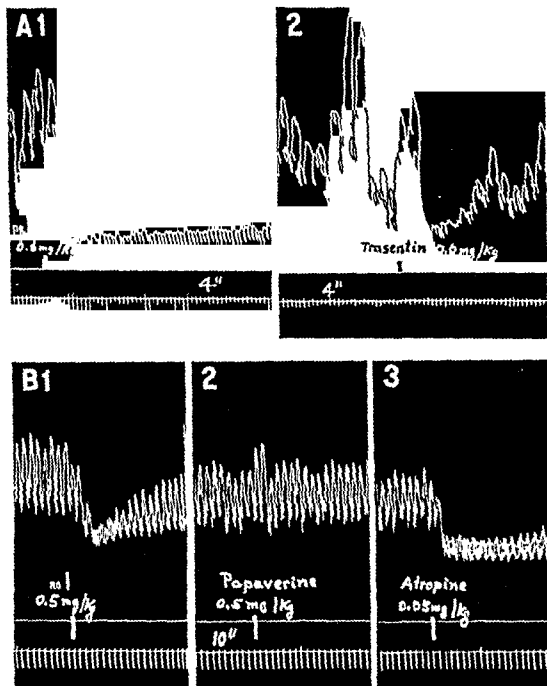


FIG. 1. GASTRIC CONTRACTIONS AFTER INSULIN IN TWO DOGS. INTRAVENOUS INJECTIONS

A. 1. Diethylaminoethyl fluorene-9-carboxylate, 0.6 mgm /kgm.

2. Diethylaminoethyl diphenylacetate, 0.6 mgm./kgm.

0.5 mgm./kgm.

3. Atropine, 0.05 mgm /kgm.

Between 1 and 2, thirteen minutes; between 2 and 3, four minutes.

per cent solution), were done on each of the four dogs. Table 2 shows the average values for each dog, and for the group, for free acid.

DISCUSSION. In a previous paper (1), the spasmolytic activity of a series of 45 compounds was measured by the ability to cause an isolated strip of intestine to relax after it had contracted intensely in response to acetyl choline, histamine, or barium chloride. The antagonism to the first of these was denoted "neurotropic" in nature, that to the second and third "musculotropic." This procedure is in accord with the practice of many others who have made similar studies. The affixing of these labels is largely gratuitous, since no direct experimental evidence supports their implications. In our experiments on the small intestine *in situ*, the potency of all compounds in depressing spontaneous motility was found to vary similarly with the potency in antagonizing spasm

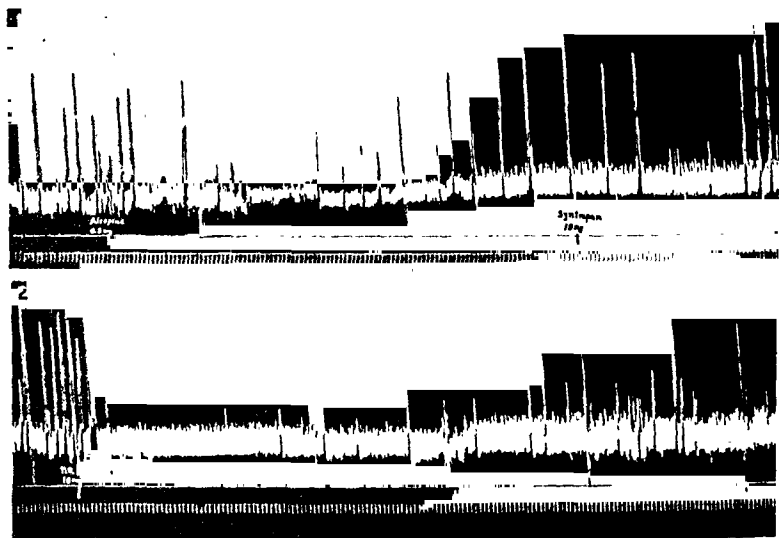


FIG. 2. GASTRIC CONTRACTIONS AFTER INSULIN IN MAN. INTRAMUSCULAR INJECTIONS

1. Atropine, 0.5 mgm., and "Syntropan," 10 mgm.
 2. Diethylaminoethyl fluorene-9-carboxylate, 10 mgm.
- Between 1 and 2, four days.

produced by acetyl choline *in vitro*. The same relationship is found in this study of gastric motility. This is reasonable, in view of the fact that gastric hypermotility produced by insulin is "central" in origin, that is, the vagus nerves must be intact for its occurrence (3). We intend to study the action of these spasmolytic substances in other organs with varying dependence on nervous structures for their motility.

CONCLUSIONS

1. Atropine and similar substances have little relaxing action on cardiospasm produced in the dog by vagotomy.

2. Atropine and similar substances reduce the gastric hypermotility produced in the dog by the injection of insulin. The comparative potency of such compounds varies similarly with their potency in depressing motility of the small

TABLE 2
Gastric emptying time in minutes

	DOG #1		DOG #2		DOG #3		DOG #4		AVERAGE	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control.....	5.3	338.7	4.0	247.0	4.0	229.7	5.7	323.0	4.8	284.6
No. 1*.....	9.0	378.7	9.3	403.3	6.7	298.3	14.0	463.0	9.8	385.9
Atropine sulfate....	14.3	487.7	17.0	444.0	9.7	390.6	12.3	518.3	13.3	462.3

Gastric secretion of free acid

	MINUTES	DOG #1 ACID	DOG #2 ACID	DOG #3 ACID	DOG #4 ACID	AVERAGE ACID
Control	15	26	20	12	13	18
	30	51	42	17	29	34
	45	66	28	30	32	39
	60	30	2	44	55	33
	75	8		74	19	24
	90			53	12	16
	105			13		3
	120			1		0
No. 1*	15	1	2	4	6	3
	30	2	1	11	16	8
	45	10	22	41	34	27
	60	11	16	60	44	33
	75	9	12	63	20	26
	90	9		67	9	21
	105	5		44	2	13
	120	3		31		11
Atropine sulfate	15	1	1	1	0	1
	30	3	2	2	0	2
	45	2	1	2	0	1
	60	1	1	2	0	1
	75	2	1	1	0	1
	90	2	1	2	0	1
	105	0	1	0	0	0
	120	0	0	0	0	0

* See table 1. Diethylaminoethyl fluorene-9-carboxylate hydrochloride.

intestine, and with their potency in antagonizing the spasmogenic effects on isolated intestine of acetyl choline, but not those of histamine or barium chloride.

3. Diethylaminoethyl fluorene-9-carboxylate hydrochloride, chosen from a

series of 44 related compounds as most promising for clinical trial, has one-twentieth the potency of atropine in reducing gastric hypermotility.

4. Diethylaminoethyl fluorene-9-carboxylate hydrochloride has much less effect than atropine in delaying gastric emptying and in reducing gastric secretion, when given in 100 times the dose.

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THE DIGITALIS-LIKE PRINCIPLES OF CALOTROPIS COMPARED WITH OTHER CARDIAC SUBSTANCES¹

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Calotropis is a genus of the family *Asclepiadaceae*, two species of which, *C. procera* and *C. gigantea*, are now known to contain digitalis-like principles. These two plants are shrubs of moderate size, six or more feet high, and give rise to a milky secretion when pricked or injured. *C. procera* is indigenous to the upper Provinces of India, Persia, Arabia, Egypt, Abyssinia, and Sudan, while *C. gigantea* grows in the southern parts of India, Ceylon, the Malayan Peninsula, and the Moluccas (2). The colloquial name of *C. procera* is "Mudar" or "Madar" in India, and "Uschar" in Arabia.

The root-bark and milky juice of both species have been used in India for the treatment of syphilis, leprosy, dysentery, elephantiasis, and other ailments (3-5). Owing to their toxic properties, the same have been employed for homicidal and suicidal purposes (4). The natives of Arabia and Africa have made use of the milky sap for arrow poisons (6-8). Duncan (3) recognized the emetic action of the root-bark, and recommended it as a substitute for ipecac in medicine.

Repeated chemical investigations have been carried out. Warden and Waddell (4) separated from the root-bark a white crystalline product, "Madar alban," m.p. 139°C., $C_{17}H_{18}O$, and a yellow resin, "Madar fluavil," m.p. 43°C., $C_{18}H_{22}O_4$. Duncan (3, 9), on the other hand, postulated the presence of "Mudarin" which he believed to be analogous to or identical with emetine. Hill and Sirkar (10) isolated two crystalline products from the root-bark of *C. gigantea*: namely, mudarol isovalerate, m.p. 140°C., $C_{30}H_{47}O \cdot CO_2 \cdot C_4H_9$; and akundarol isovalerate, m.p. 210°C., $C_{38}H_{61}O \cdot CO \cdot C_4H_9$.

Gerber and Flourens (11, 12), working with the milky secretion of *C. procera*, found a proteolytic enzyme and described its characteristics. Similarly, Basu and Nath (13) reported the occurrence of a proteinase in the milky juice of *C. gigantea*, and compared it with papain. The same authors in addition isolated a sterol, termed calosterol, m.p. 202-203°C., $C_{28}H_{44}O$.

Although the emetic and other toxic effects of the root-bark and latex of *Calotropis* were repeatedly observed in animals and men (3, 11, 12, 15), it remained for Lewin (6) to show conclusively the digitalis-like action of the milky juice of *C. procera*. Chemists' efforts henceforth have been directed to

¹ Read by title at the New Orleans Meeting of the American Society for Pharmacology and Experimental Therapeutics, 1940 (1).

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the isolation of these active principles. Wieland (16) succeeded in isolating calotropin as a chloroform addition compound. His work was followed by that of Jacobi (16) on the same product. Both of them withheld publication. It was Hesse (16, 17) who reported the details of calotropin, m.p. 221°C ., $\text{C}_{29}\text{H}_{40}\text{O}_9$, which he obtained from the leaves and stems of *C. procera*. Subsequently, he announced the isolation of uscharin, decomp. 265°C ., $\text{C}_{31}\text{H}_{41}\text{O}_8\text{NS}$, calotoxin, decomp. 244°C ., $\text{C}_{29}\text{H}_{40}\text{O}_{10}$, and calactin from the milky sap of *C. procera* and *C. gigantea* (17). The last substance was not characterized. Of particular interest is uscharin, for unlike most cardiac glycosides it contains nitrogen and sulfur in its molecule. Through the courtesy of Dr. Gerhard Hesse, Munich and Marburg, we have had a supply of calotropin, calotropagenin, calotoxin, and uscharin, and found all of them to have a digitalis-like action—thus confirming Hesse's findings. Results in cats and frogs with calotropin and calotropagenin were presented in two previous publications (18, 19).

The present study was chiefly concerned with the relative potency of calotropin, calotoxin, and uscharin in comparison with ouabain and other cardiac substances. The necessity of testing standard preparations in parallel with samples of unknown potency is well recognized in biological assay. It is less generally appreciated, perhaps, that the same variations in mean susceptibility may occur in investigations on new drugs. Unless older, well-known compounds are retested at the same time as the newer substances, it is impossible to determine how much of an observed difference in the LD_{50} is due to a difference in drug potency and how much to a greater or lesser susceptibility in the given lot of test animals. In the present experiments ouabain served as a "standard," both in determinations with frogs and in those with cats. To examine more fully the importance of this precaution in experiments with cats, eight additional cardiac principles were included as well in a single comprehensive experiment.

RELATIVE TOXICITIES IN FROGS. The comparisons in frogs were made by the U.S.P. one-hour method (20). Because frogs vary from one assay to another in their susceptibility to digitalis, the Pharmacopoeia requires that the reference standard be retested with each sample. The same condition has been observed here. Ouabain served as the standard and calotropin, calotoxin, and uscharin as the unknowns, in an experiment run at the same time on a single lot of animals.

The results in table 1 have been computed by methods that are described elsewhere (21). The dosage-effect curves for the four drugs, plotted as straight lines in terms of probits and the logarithm of the dose, had a combined slope of $b_c = 17.9 \pm 2.7$. The values for the individual drugs agreed sufficiently to consider the four dosage-effect curves as parallel and the median systolic doses and their standard errors ($\text{SD}_{50} \pm \text{S.E.}$) have been computed on this basis. The SD_{50} 's for the four drugs may be compared with one another within the series, using the standard errors to evaluate the differences between

them. Their relative potencies in frogs, all differing significantly from one another, are listed below:

DRUG	IN ORIGINAL FORM	IN ANHYDROUS FORM
Ouabain.....	100	100
Calotropin.....	84.4 \pm 3.3	69.2 \pm 2.7
Calotoxin.....	62.9 \pm 2.4	50.8 \pm 1.9
Uscharin.....	35.0 \pm 1.4	28.0 \pm 1.1

TABLE 1

Assay in frogs, in terms of original weights of drug

COMPOUND	SOLUTION	DOSE	NUMBER IN SYSTOLE/NUMBER OF FROGS USED	MEDIAN SYSTOLIC DOSE (SD \pm S.E.)
		<i>µm. per gram</i>		<i>µm. per gram</i>
Ouabain	1:10,000	0.8	1/10	1.047 \pm 0.026
		0.9	3/10	
		1.0	5/20	
		1.1	6/10	
		1.2	8/10	
Calotropin	1:10,000	1.0	0/10	1.240 \pm 0.038
		1.1	0/10	
		1.2	6/10	
		1.3	7/10	
Calotoxin	1:5,000	1.3	0/10	1.665 \pm 0.049
		1.6	2/10	
		1.7	7/10	
		1.8	8/10	
Uscharin	1:5,000	2.7	2/10	2.992 \pm 0.091
		3.0	5/10	
		3.2	7/10	
		4.0	10/10	
		5.0	10/10	

RELATIVE TOXICITIES IN CATS. *Experimental procedure.* The relative toxicities in etherized cats of the digitalis-like principles of *Calotropis*, calotropin, calotoxin, and uscharin, of ouabain, and of eight other cardiac principles were determined simultaneously, every drug being tested on one cat on each of the twelve days of the experiment. Six cats were run at 10:30 a.m. and six at 2:30 p.m. by three observers, each injecting two cats at the same time. During the experiment every drug was tested six times in the morning and six times in the afternoon and twice during each period by each of the three observers. In this way any differences in the just-toxic dose associated with days, with the morning or afternoon, or with observers were so balanced that they could not bias comparisons between drugs. To meet these requirements, the rows and columns of the 12 x 12 Latin square given by Fisher and Yates (22) were randomized to obtain the design shown in table 2. The columns in table 2 represent the 12 dates of the experiment, noted across the top, the rows the

time of day and observer as indicated in the left margin, and the letters A to L the 12 drugs, which are listed in alphabetical order in table 3 with the results on the individual cats for each day.

The principle of the assay method was that of Hatcher and Brody (23). A 1:20,000 dilution of each of the 12 drugs was freshly prepared daily from a 0.1 per cent stock solution containing 47.5 per cent alcohol, and injected into a femoral vein at the rate of 1 cc. per minute. Death was determined by means of a stethoscope, and the injection was terminated as soon as the heart sound disappeared. Ether was employed as the anesthetic.

Since death is due to cardiac arrest, the toxic dose may be related more closely to heart weight than to body weight. Although heart weight increased proportionately with the body weight in both males and females in a series of 327 cats used in cardiac assays, their hearts after careful dissection weighed from 0.30 to 0.60 per cent of the total body, averaging 0.41 per cent. The distribution of percentage heart weights over this range is shown in

TABLE 2
Randomized design for the cat assay (see text)

TIME	OBSERVER	DATE											
		3/6	3/7	3/8	3/9	3/13	3/14	3/16	3/21	3/24	3/27	3/30	4/3
10:30 a.m.	I	I	J	B	L	H	G	F	K	D	E	A	C
		K	G	J	H	I	B	L	C	E	F	D	A
	II	B	L	G	C	D	J	K	E	H	A	F	I
		E	D	F	G	J	K	A	L	C	I	B	H
	III	C	K	A	B	F	L	I	D	G	H	J	E
		F	H	K	E	G	C	D	B	A	L	I	J
	I	J	C	E	K	A	I	H	F	B	G	L	D
		D	F	I	A	L	E	C	G	J	B	H	K
	II	A	B	C	D	E	F	G	H	I	J	K	L
		H	E	L	J	C	A	B	I	K	D*	G	F
2:30 p.m.	III	G	I	D	F	K	H	J	A	L	C	E	B
		L	A	H	I	B	D	E	J	F	K	C	G

* This cat aberrant and replaced by another run on 3/28.

figure 1. To test whether the use of heart weight rather than body weight would permit a more sensitive discrimination between the cardiac substances in the present series, the heart was also dissected out upon the death of each cat and weighed.

One cat in the original series, that given calotoxin on 3/27, died soon after the beginning of injection, presumably due to asphyxia. This cat was omitted, therefore, from all calculations and in the final estimates of the median lethal dose replaced by a new cat tested on 3/28 as listed in table 3.

Analysis of the data. Briefly, all basic computations were made in terms of logarithms since logarithmic units vary more uniformly and symmetrically than arithmetic units (24). It was revealed that the comparison would be most precise if the dose were related to the heart weight rather than to the body weight. Further, in adjusting dosage for size of the heart, the weight of the heart was raised to the $\frac{2}{3}$ power (25).

TABLE 3
Original data for the cat assay in table 2

DRUG	SYMBOL IN TABLE 2	DATE	SEX	BODY WEIGHT	HEART WEIGHT	FATAL DOSE BASED ON	
						Body weight	Heart weight
				kgm.	grams	$\mu\text{gm. per kgm.}$	$\mu\text{gm. per gram}$
α -Antiarin	A	3/6	F	1.969	6.5	103.4	31.3
		3/7	F	2.561	11.0	95.2	22.2
		3/8	F	2.405	9.5	104.4	26.4
		3/9	F	1.938	7.9	109.3	26.8
		3/13	M	2.270	9.6	139.6	33.0
		3/14	M	2.818	10.1	102.3	28.5
		3/16	F	2.109	8.1	84.2	22.0
		3/21	M	1.938	8.1	82.9	19.8
		3/24	F	1.939	7.4	85.3	22.3
		3/27	F	1.934	8.6	120.5	27.1
		3/30	F	2.168	7.6	71.9	20.5
		4/3	M	2.349	8.5	73.4	20.3
β -Antiarin	B	3/6	F	2.063	7.7	95.1	25.5
		3/7	M	2.091	7.0	94.3	28.1
		3/8	F	2.103	7.8	98.3	26.5
		3/9	M	2.644	13.6	120.1	23.4
		3/13	F	2.649	9.9	109.4	29.2
		3/14	F	2.747	13.2	81.4	17.0
		3/16	F	1.908	7.2	85.3	22.6
		3/21	M	1.955	6.7	83.2	24.3
		3/24	M	1.923	9.1	93.1	19.7
		3/27	F	1.892	8.0	98.4	23.3
		3/30	M	2.481	9.3	89.7	23.9
		4/3	M	1.769	5.8	83.1	25.3
Bufotalin	C	3/6	F	1.946	8.1	205.2	49.3
		3/7	F	2.195	7.4	157.9	46.8
		3/8	M	2.273	8.8	122.8	31.8
		3/9	F	2.422	10.7	106.7	24.2
		3/13	F	2.552	10.0	104.5	26.7
		3/14	F	1.700	7.1	163.6	39.1
		3/16	M	2.139	7.2	155.9	46.3
		3/21	F	2.050	7.3	78.9	22.2
		3/24	M	2.521	8.2	148.4	45.6
		3/27	F	2.404	9.7	178.6	44.2
		3/30	M	2.002	7.5	139.9	37.3
		4/3	M	1.868	8.0	121.9	28.6
Calotoxin	D	3/6	F	2.134	8.5	96.5	24.2
		3/7	M	2.652	10.5	103.2	26.0
		3/8	F	2.356	8.7	190.3	51.5
		3/9	M	2.461	8.7	88.0	24.9
		3/13	F	2.283	10.8	143.3	30.3
		3/14	M	2.548	10.1	91.9	23.1
		3/16	M	2.353	9.6	105.5	25.8
		3/21	M	1.821	7.3	136.0	34.0
		3/24	F	1.873	6.7	78.2	21.8
		3/28	F	1.955	6.6	99.6	29.5

TABLE 3—Continued

DRUG	SYMBOL IN TABLE 2	DATE	SEX	BODY WEIGHT	HEART WEIGHT	FATAL DOSE BASED ON	
						Body weight	Heart weight
				kgm.	grams	μgm. per kgm.	μgm. per gram
Calotropin	E	3/6	F	2.039	8.7	97.7	22.9
		3/7	M	2.421	9.1	108.4	28.8
		3/8	F	2.547	9.3	87.9	24.1
		3/9	M	2.372	9.0	119.0	31.4
		3/13	F	2.474	9.9	90.3	22.6
		3/14	F	2.604	11.2	121.7	28.3
		3/16	F	2.240	9.6	99.5	23.2
		3/21	M	1.825	6.1	109.7	32.8
		3/24	F	1.904	9.3	113.6	23.3
		3/27	M	1.864	7.2	96.2	24.9
		3/30	M	1.792	7.1	121.4	30.6
		4/3	F	2.256	9.0	78.3	19.6
Convallotoxin	F	3/6	F	1.703	5.4	63.6	20.1
		3/7	F	2.755	8.6	69.8	22.4
		3/8	F	2.306	10.0	111.6	25.8
		3/9	F	2.541	11.4	90.3	20.1
		3/13	M	2.668	11.6	90.9	20.9
		3/14	M	2.505	10.4	76.7	18.4
		3/16	F	2.374	7.5	47.7	15.1
		3/21	M	1.735	6.5	77.0	20.6
		3/24	F	1.899	7.4	72.7	18.6
		3/27	F	1.768	5.8	59.5	18.1
		3/30	M	1.881	7.3	83.8	21.6
		4/3	F	2.188	9.9	69.5	15.3
Coumagine HCl	G	3/6	M	1.983	7.8	116.5	29.6
		3/7	F	2.014	9.3	110.0	23.8
		3/8	F	2.132	8.3	110.2	28.3
		3/9	F	2.284	8.4	109.9	29.9
		3/13	F	1.766	6.6	135.3	36.2
		3/14	F	1.952	8.3	86.6	20.4
		3/16	F	2.269	9.7	92.6	21.6
		3/21	F	2.793	11.9	90.4	21.2
		3/24	F	2.725	8.4	102.0	33.1
		3/27	M	2.516	9.6	86.2	22.6
		3/30	F	1.771	6.7	142.6	37.7
		4/3	F	1.841	7.0	135.8	35.7
Cymarín	H	3/6	F	2.196	9.4	83.1	19.4
		3/7	F	2.054	8.7	110.2	26.0
		3/8	F	2.430	9.2	87.3	23.0
		3/9	F	2.661	9.9	100.1	26.9
		3/13	F	1.615	7.1	95.7	21.8
		3/14	F	2.375	8.6	91.2	25.2
		3/16	F	2.072	7.8	87.1	23.1
		3/21	F	2.032	7.5	117.2	31.7
		3/24	F	2.247	8.5	82.0	21.7
		3/27	F	2.297	7.4	103.8	32.2
		3/30	F	2.446	7.6	99.7	32.1
		4/3	F	1.791	6.4	94.5	26.4

TABLE 3--Concluded

DRUG	SYMBOL IN TABLE 2	DATE	SEX	BODY WEIGHT	HEART WEIGHT	FATAL DOSE BASED ON	
						Body weight	Heart weight
Emicymarin	I	3/6	F	2.285	8.9	146.7	37.6
		3/7	F	2.328	10.3	173.1	39.2
		3/8	M	2.278	8.2	97.8	27.2
		3/9	M	2.419	10.8	203.5	45.6
		3/13	F	2.218	10.1	125.3	27.5
		3/14	F	2.414	11.7	195.7	40.4
		3/16	M	2.174	8.9	98.2	24.0
		3/21	M	1.969	8.1	113.1	27.5
		3/24	M	2.053	8.1	145.9	37.0
		3/27	F	2.121	7.8	97.6	26.5
		3/30	F	1.812	6.5	163.8	45.7
		4/3	M	2.273	9.2	144.1	35.6
Ouabain	J	3/6	M	2.105	8.3	112.9	28.6
		3/7	M	2.573	10.9	72.8	17.2
		3/8	F	1.827	6.7	85.7	23.4
		3/9	F	2.197	9.4	106.2	24.8
		3/13	M	1.680	7.0	85.8	21.3
		3/14	M	2.820	10.9	72.0	18.6
		3/16	F	2.093	8.9	73.1	17.2
		3/21	M	2.047	8.6	100.7	23.9
		3/24	M	2.440	9.6	64.8	16.5
		3/27	F	2.319	9.1	87.1	22.2
		3/30	F	2.167	7.1	93.6	28.6
		4/3	M	1.809	7.7	95.7	22.5
Periplocymarin	K	3/6	M	2.653	11.9	205.8	45.9
		3/7	F	2.022	8.5	123.6	29.4
		3/8	M	1.831	7.4	132.2	32.7
		3/9	F	2.310	7.3	140.7	44.5
		3/13	F	1.808	8.6	156.9	33.0
		3/14	F	2.252	10.0	203.4	45.8
		3/16	F	2.263	9.8	123.5	28.5
		3/21	F	2.104	7.4	141.4	40.2
		3/24	M	2.611	9.6	199.0	54.1
		3/27	F	2.460	9.6	111.6	28.6
		3/30	F	1.773	7.3	252.1	61.2
		4/3	F	2.225	9.2	133.7	32.3
Uscharin	L	3/6	F	2.187	8.8	151.6	37.7
		3/7	F	2.169	8.4	123.3	31.8
		3/8	M	2.275	8.7	185.3	45.4
		3/9	F	2.309	8.2	156.3	44.0
		3/13	M	1.906	8.0	180.5	43.0
		3/14	M	1.969	7.7	140.9	36.0
		3/16	F	2.207	9.7	163.3	37.2
		3/21	M	2.570	9.9	109.3	28.4
		3/24	F	2.222	8.5	123.3	32.2
		3/27	F	2.169	6.5	127.5	42.5
		3/30	M	2.325	8.9	101.5	26.5
		4/3	F	1.802	7.1	210.9	53.5

Several other factors in the present experiment have been balanced in the design as insurance to prevent their biasing our estimates of relative potency. The amount of variation excluded by this precaution can be measured readily by the analysis of variance (26), which has been computed in logarithmic units for both the customary $\mu\text{gm.}$ of drug per kilogram of body weight and

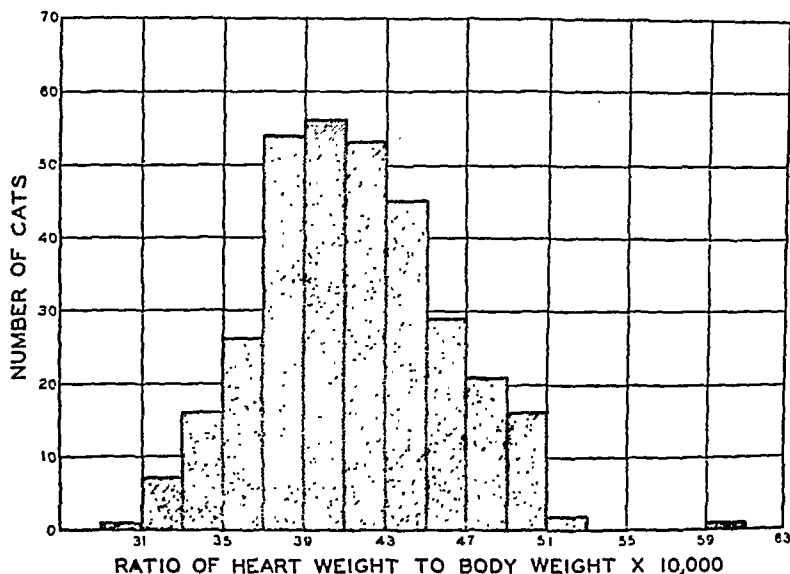


FIG. 1. DISTRIBUTION OF RATIO OF HEART WEIGHT TO BODY WEIGHT ($\times 10,000$) IN 141 MALE AND 186 FEMALE CATS

TABLE 4

Comparison of sources of variation in the cat assay in tables 2 and 3 by the analysis of variance, in terms of the fatal dose per kgm. of body and of the fatal dose per (gram) $\frac{1}{3}$ of heart, both expressed in logarithmic units

VARIATION	DEGREES OF FREEDOM	BASED ON BODY WEIGHT		BASED ON HEART WEIGHT	
		Mean square	F	Mean square	F
Between days.....	11	.01216	1.46	.01254	1.64
A.M. vs. P.M.....	1	.01159	1.39	.01722	2.25
Between operators.....	2	.01703	2.04	.01592	2.03
Between drugs.....	11	.08678	10.42	.08144	10.65
Composite error.....	117	.005331	1.00	.007645	1.00

$\mu\text{gm.}$ of drug per "surface unit" of heart or (weight of heart) $^{\frac{1}{3}}$. Table 4 shows for each component at the left the degrees of freedom, mean square of the deviations and the ratio (F) of these mean squares to the composite experimental error. The reduction in the experimental error when dosages were corrected for size of heart rather than for body weight is evident. The varia-

tion between days over the relatively brief period of less than a month, between morning and afternoon and between operators in the same laboratory, exceeded the experimental error, so that the experiment gained precision by segregating these factors even though they were not statistically significant. On the basis of their original weights, the drugs differed very significantly in potency.

Logarithms have been used in comparing drugs in part because the mean log-dose is an estimate of the median or log-LD₅₀ and its antilogarithm the geometric mean or LD₅₀. Whether drug potencies can be compared satisfactorily in terms of the LD₅₀'s alone depends upon the several drugs agreeing with each other in their variability as measured by the standard deviation of the individual log-dose. When adjusted for heart size, as in the second part of table 4, but including effects of date, hour, and operator, the standard deviations for the separate drugs varied from 0.057 to 0.132. Yet a chi-square test for the series as a whole indicated no significant difference ($P = .12$) from their mean, $s = 0.0905$. The use of a single standard deviation for all drugs thus having been justified, the appropriate value for their comparison was that based upon the "composite error" in table 4, excluding effects of date, hour, and operator, or $s = \sqrt{.007645} = 0.0874$. In terms of this standard deviation, a difference between the log-LD₅₀'s for any two drugs of less than (0.0874) (1.981) $\sqrt{2/12} = 0.0707$ could not be considered significant, a difference between 0.0707 and 0.0843 should be viewed as doubtfully significant, and only those greater than 0.0843 can be regarded as established differences in potency. When potencies from a large group selected at will were compared at the end of the experiment, the difference corresponding exactly to odds of 1 in 20, the usual level of significance, has not been solved by the mathematical statisticians, so that values between apparent odds of $P = .05$ and $P = .02$, as above, would be considered "doubtfully significant."

Relative potencies in cats. Most reports on the potency of cardiac principles in cats have been stated in units of drug per kilogram of body weight. The geometric mean computed from such values is an estimate of the dose killing 50 per cent of the cats or the LD₅₀. The second column of table 5 gives the LD₅₀ and its standard error in $\mu\text{gm.}$ per kilogram of body weight for the 10 drugs in this series reported previously from this laboratory (27-30), each based on 10 to 37 cats but covering a period of several years. The next column of the table shows the LD₅₀'s obtained simultaneously during the present experiment, with standard errors computed separately for each drug from the dosages in table 3. In both series the potencies are in terms of the original (not anhydrous) weight of drug. For half of the drugs the present determination of the LD₅₀ does not differ significantly from that reported previously, but for four drugs the two determinations differ significantly and in the other case the difference is nearly significant. A discrepancy of this magnitude demonstrates that determinations of the lethal dose in cats, even when made by experienced workers in the same laboratory, are not independent of changes in the level of susceptibility as has generally been assumed. One

would place considerably more reliance, therefore, in a ranking of potencies based upon the present experiment.

Since several of the compounds contained water or alcohol of crystallization, they were dried by heating in a high vacuum to constant weight and the loss in weight determined. Three of the compounds, coumagine HCl, periplocymarin, and uscharin, were already in an anhydrous state, but the remaining nine lost from 0.14 to 19.87 per cent in weight on drying, as shown in the fourth column of table 5. Periplocymarin when freshly prepared contained a molecule of alcohol of crystallization (30), but it apparently had lost the latter on standing. The determinations of LD_{50} have been corrected, therefore, to an anhydrous form and are given in $\mu\text{gm.}$ per kilogram of body weight in the fifth column of the same table.

TABLE 5

Median lethal doses (LD_{50}), and relative potencies in logarithms (M) and in percentages for 12 cardiac drugs, computed from data reported previously (2d column) and from those in table 3 (remaining columns)

DRUG	LD_{50} BASED ON BODY WEIGHT (WITH SOLVENT OF CRYSTALLIZATION)		LOSS OF WEIGHT ON DRYING	LD_{50} IN ANHYDROUS FORM BASED ON		
	Old data	New data		Body weight	Heart weight	
	$\mu\text{gm. per kgm.}$	$\mu\text{gm. per kgm.}$		$\mu\text{gm. per kgm.}$	M	per cent
Convallootoxin.....	75.5 ± 3.7	81.1 ± 5.2	8.21	74.4 ± 4.8	.0471	111.5
Ouabain.....	119.1 ± 3.0	106.8 ± 5.4	19.87	85.6 ± 4.4	0	100.0
β -Antiarin.....	102.0 ± 5.8	102.2 ± 3.5	8.33	93.7 ± 3.2	-.0443	90.3
α -Antiarin.....	128.2 ± 4.3	99.1 ± 5.7	3.26	95.9 ± 5.5	-.0592	87.3
Cymarin.....	125.2 ± 3.8	97.8 ± 3.1	2.45	95.4 ± 3.0	-.0666	85.8
Calotropin.....	119.3 ± 3.0	105.2 ± 4.3	2.29	102.8 ± 4.2	-.0810	83.0
Coumagine HCl..	145.4 ± 9.4	108.2 ± 5.6	0	108.2 ± 5.6	-.1076	78.1
Calotoxin.....		112.3 ± 10.6	0.70	111.5 ± 10.5	-.1177	76.3
Emicymarin.....	161.8 ± 13.0	143.7 ± 11.0	4.25	137.6 ± 10.5	-.2019	62.8
Bufotalin.....	126.6 ± 9.2	136.2 ± 10.4	0.14	136.0 ± 10.4	-.2140	61.1
Uscharin.....		144.5 ± 9.3	0	144.5 ± 9.3	-.2400	57.5
Periplocymarin....	149.9 ± 6.1	155.3 ± 11.6	0	155.3 ± 11.6	-.2571	55.3

As has been shown, heart weight gave a more adequate adjustment for differences in the size of cats than the weight of the body, leading to a smaller composite error. Hence the $\mu\text{gm.}$ per unit of heart "surface" gave the most precise ranking of potency for the 12 drugs. These are listed in the last two columns of table 5, where ouabain has been used as a standard. The log-ratios of potency, M , in the first of the last two columns show that calotropin and all succeeding drugs in the list have a significantly smaller potency than ouabain. Any two drugs may be compared with each other, however, by subtracting their values of M , the antilogarithm of the difference measuring their relative potencies. Differences between selected values of M which do not exceed ± 0.0707 cannot be considered as significant and only those larger than ± 0.0843 as certainly significant. Comparison of the percentage potencies in the last column with corresponding values based upon body weight shows that the two

series agree within a few per cent in every case, and in only two pairs of drugs, which had almost the same toxicities, was the order reversed between the two criteria.

SUMMARY

1. Of the three digitalis-like principles of *Calotropis*, their potencies relative to each other and to ouabain were in the following order when tested in parallel:

DRUGS IN ANHYDROUS FORM	IN Frogs	IN CATS
Ouabain.....	144	121
Calotropin.....	100	100
Calotoxin.....	76	92
Uscharin.....	42	69

All differences were significant except that between calotropin and calotoxin in cats.

2. By simultaneous comparison of the above 4 drugs with 8 other cardiac principles in etherized cats, their potencies in anhydrous form relative to ouabain were convallotoxin 112, ouabain 100, β -antiarrin 90, α -antiarrin 87, cymarin 86, calotropin 83, coumagine HCl 78, calotoxin 76, emicymarin 63, bufotalin 61, uscharin 58, and periplocymarin 55. Differences between pairs in the above series that are less than 18 per cent of the smaller potency cannot be considered significant.

3. For 10 of the above drugs, the median lethal dose had also been determined individually over a period of several years and in four cases the new values differed significantly from the earlier determinations, demonstrating the importance of parallel tests with group standards in experiments on the toxicity of cardiac substances to cats.

4. The lethal dose in cats showed a closer relation to the size of the heart, in terms of the $\frac{2}{3}$ power of its fresh weight, than to the weight of the body, so that comparisons of drugs corrected for size of heart were the more precise and have been quoted in the summary. Due to the high correlation between heart weight and body weight, relative potencies determined by both procedures agreed within a few per cent.

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A STUDY OF ANTIDOTES FOR FLUORINE

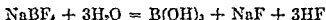
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Sodium fluoride is commonly used as a roach powder. When left around the kitchen it is sometimes mistaken for baking powder. This fact received considerable publicity (1) recently when 11 men died and 52 became ill from eating pancakes made with roach powder. The sodium fluoride was mistaken for baking powder at the Salvation Army in Pittsburgh. From 1 to 2 teaspoonfuls of baking powder per cup of flour is commonly used in pancakes. It is thus possible for one to get from 5 to 10 grams of sodium fluoride, which is considered a fatal dose. To prevent sodium fluoride from being mistaken for flour, sugar, or baking powder, some manufacturers are now coloring this insecticide a Nile blue. As a further precaution the National Pest Control Association suggests the importance of knowing more about the antidotes for fluorine and the possibility of mixing these with the fluoride. Studies were therefore made at the Tennessee Agricultural Experiment Station with these suggestions in view.

THE FLUOBORATES. The past season an opportunity presented itself for testing sodium fluoborate (NaBF_4) as an insecticide. Much to our surprise this compound showed no toxicity to insects when fed in the powdered form, although it contains 68 per cent soluble fluorine. This was tested further on white rats. Sherman B diet was used and sodium fluoborate added to produce 0.1 per cent fluorine. The animals made normal gains in weight on the fluoborate diet, which again proved non-toxic. It appears that in NaBF_4 the BF_4 ion is but slightly dissociated. The controls given sodium fluoride lost weight steadily.

In the next series tests were conducted to see if small amounts of the fluoborate in the drinking water would cause striations on the teeth. Solutions of sodium fluoborate and potassium fluoborate were made up to contain 11 parts of fluorine per million. A stock solution of one gram in 400 cc. of water was used. At the end of 5 weeks the teeth of the rats were distinctly striated. In the course of the work the fluoborates were found to undergo slow hydrolysis when dissolved in water, probably in accordance with the following equation:

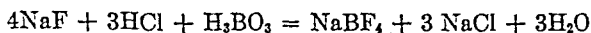


Sodium fluoborate in a one per cent solution shows a pH of 3.6. Just after the solution is prepared 0.05 cc. of 0.1 normal NaOH will neutralize 1 cc. of it.

After the solution has stood for 9 days 1 cc., and after 45 days 1.5 cc. of 0.1 normal NaOH will be required to neutralize 1 cc. of sodium fluoborate. A steady hydrolysis is thus seen to take place. A one per cent solution of potassium fluoborate shows a pH of 6.0 and hydrolysis takes place somewhat more slowly in it.

Another series of rats were given potassium fluoborate in the drinking water at the rate of 7 p.p.m. of fluorine. The solution was prepared each day to prevent hydrolysis. At the end of 5 weeks the teeth showed no striations, while the control rats, with 7 p.p.m. fluorine as sodium fluoride, showed distinct striations.

Since the fluoborates possess a complex ion in which the fluorine is tied up, it was thought that boron or boric acid might act as an antidote for fluorine in accordance with the following equation:



This reaction does not go to completion in the presence of water, but may do so when the solution is heated sufficiently to drive off the water. Nevertheless considerable fluoborate is found even in the presence of water. A stock solution was made up as follows:

NaF.....	1 gram
H ₃ BO ₃	0.5 gram
HCl (Conc.).....	2.8 cc.
Water.....	100 cc.

One cc. of HCl alone as above neutralized 3.5 cc. of 0.1 normal NaOH, but when used in the above formula 1 cc. neutralized only 1.65 cc., indicating that considerable fluoborate was formed.

This stock solution was diluted to contain 1000 p.p.m. sodium fluoride and used at this rate in the drinking water. The animals lived 27 days, whereas the controls on sodium fluoride (1:1000) lived but 3 days. When boric acid only was added to the sodium fluoride the rats lived 5 days. This experiment would indicate that the combination of HCl and H₃BO₃ added to the sodium fluoride tends to form some fluoborate, which in turn is less toxic than the sodium fluoride. Boric acid may thus be considered to possess some value as an antidote.

In the next series of tests the above combinations were diluted to contain 7 p.p.m. of fluorine to note the effects on the teeth. At this high dilution the fluoborates are more completely hydrolyzed, so that no corrective effect of the boric acid could be noted as far as the striations on the teeth were concerned.

FEEDING TESTS WITH CALCIUM AND ALUMINUM COMPOUNDS. The most commonly accepted antidote for fluorides is calcium in some form. Recently Sharples (2) showed that aluminum chloride possesses the power of limiting fluorine toxicosis in the rat. With 0.1 per cent sodium fluoride his animals grew at two-thirds the normal rate. By adding a solution of 0.5 per cent aluminum chloride or 2 per cent calcium carbonate to the diet, he obtained normal growth.

In our work at the Tennessee Station the following materials were fed: calcium hydroxide, magnesium hydroxide, calcium lactate, calcium phosphate, bonemeal, boric acid, borax, aluminum sulphate, activated alumina, aluminum powder, bauxite, clay. The sodium fluoride was fed at a level of 0.2 per cent. At this rate the animals lost weight and lived approximately a month. Larger amounts of sodium fluoride were not well tolerated and the animals refused to eat much, even when starved.

The best results were obtained with the aluminum sulphate— $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$. With this material at the rate of 0.4 per cent and sodium fluoride 0.2 per cent in the diet, the animals were able to gain 68 per cent in growth when compared with the controls, which had no antidote. The insoluble activated alumina Al_2O_3 also did well by permitting a 60 per cent gain. Borax showed 53 per cent gain and lime 49. Bonemeal and calcium phosphate were both very poor, with a gain of only 6 per cent.

In another series both calcium lactate and magnesium hydroxide were found to be inferior to calcium hydroxide. In still another experiment 0.8 per cent aluminum powder, such as is used in making aluminum paint, showed a 7 per cent gain over the initial weight, while 0.1 per cent showed a loss of 10 per cent in weight. Eight-tenths per cent bauxite, as well as 0.8 per cent clay, was of little value.

TESTS ON RABBITS WITH FATAL DOSES OF SODIUM FLUORIDE. The minimum lethal dose of sodium fluoride for a rabbit was found by Muehlberger (3) to be 200 mgm. per kilogram of body weight. The sodium fluoride was administered by means of a stomach tube, presumably in solution. The length of time the rabbits lived after receiving a barely lethal dose was 5-7 days. Other investigators give an M.L.D. of 500 mgm. per kilogram. When this dose is given in solution by stomach tube, death results in about 1 hour (4, 5).

In our work the animals were kept for 24 hours without food. The sodium fluoride was then given in powdered form in capsules at the rate of 500 mgm. per kilogram. With this dose the rabbits lived only from one to three hours. The same dose was employed in subsequent tests and mixed with the antidote dry and also administered as capsules. When aluminum sulphate was employed as the antidote in a ratio of 2 parts to 1 part of sodium fluoride, the rabbit remained alive. Activated bauxite was equally successful when used at the same rate. Powdered calcium hydroxide (C.P.) testing 87 per cent, and with 13 per cent as calcium carbonate, also prevented death when used in a ratio of 1 to 1 with sodium fluoride. When the aluminum sulphate was mixed in a ratio of 2 parts of sodium fluoride to 1 part of antidote, so that the mixture contained 33 per cent aluminum sulphate, the animal lived 5 days. With 20 per cent aluminum sulphate, the rabbit lived 4 days, while with 11 per cent death resulted in 45 minutes.

EFFECT ON INSECTS. Sodium fluoride has proved to be the best insecticide in roach control. About 25 million pounds are used annually for this purpose in the United States.

In the tests on insects, sodium fluoride was employed as a powder and dusted

on the roaches. After the roaches were dusted with measured amounts of sodium fluoride they were placed in cages to determine the per cent mortality. When antidotes such as boric acid, aluminum sulphate, aluminum oxide, or lime were mixed as powders with the sodium fluoride, the control obtained was definitely lowered. Even when the above materials were employed as coarse powders, the kill obtained was less than with the sodium fluoride alone. This phase of the subject, using the antidote in large particle form as compared with the finely divided sodium fluoride, is being investigated. It is assumed that the aluminum sulphate for example, even though coarse, will dissolve in the stomach of man. The roach, however, picks up the finely divided sodium fluoride in dry form.

SUMMARY

The fluoborates when fed in powdered form were found to be non-toxic to both rats and insects. In solution enough fluorine is liberated to produce toxic effects.

Both aluminum sulphate and hydrated lime are capable of saving animals from a lethal dose of sodium fluoride when mixed with the latter. Boric acid also has some value as an antidote through the formation of a fluoborate. The aluminum sulphate forms cryolite which was found to be much less toxic. When aluminum sulphate is mixed with the sodium fluoride and used as a roach powder, a lower kill is obtained than with sodium fluoride alone.

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THE MECHANISM OF MORPHINE MIOSIS

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Modern views of morphine miosis have largely been based on the work of Picard (1, 2), who removed the roof of the skull of a morphinized dog, elevated the brain, cut the optic nerve, and observed no change in the original degree of miosis. He then sectioned the cerebral hemispheres from the front progressively backwards without observing any pupillary change until the anterior median portion of the cerebral peduncles was reached. Section at this point caused an abrupt mydriasis. From these experiments it was concluded that morphine acts by stimulating certain cells within the brain, presumably the oculomotor center. Henderson and Graham (3) reached an essentially similar conclusion. Using decorticate dogs they found that miosis changed to mydriasis on injury to or section of the anterior corpora quadrigemina, and that unilateral injury produced ipsilateral mydriasis. They concluded that morphine miosis depends on direct stimulation of these structures although action on the oculomotor center was not entirely excluded.

An alternative suggestion appears to have been first made by Braunstein (cf. Starkenstein, 4), and later adopted by Meyer and Gottlieb (5). It was based on the idea that the action of morphine was always to produce paralysis; and since paralysis of the sympathetic was excluded,¹ it was necessary to assume that the paralysis affected inhibitory stimuli to the oculomotor nucleus, and these, it was supposed, arose in the cortex. The work of Henderson and Graham (3) renders this extremely unlikely, for they found that removal of the cortex, and consequently removal of such an inhibitory center, does not produce any very great degree of miosis in the absence of morphine.

We are left, then, with the hypothesis of a direct central stimulation, the main evidence for which is due to Picard. His experiment, however, was an acute one and may have involved considerable injury to the brain; moreover, he did not demonstrate any effect on the pupil in the absence of morphine.

Effect of severing the optic nerve. In order to sever the optic nerve without at the same time cutting the fibers of the oculomotor nerve which run in its sheath (6), the following operation was performed. Dogs were anesthetized with morphine-ether. A short incision was made with aseptic precautions at the outer canthus of the left eye; a small triangular opening was then made at the outer

¹ The opposite view was taken by Amsler (6). On the basis of finding that morphine produced nearly the same degree of miosis when the cervical sympathetic was cut, and did not produce miosis after removal of the cerebral hemispheres, he concluded that inhibition of an antagonistic cortical sympathetic inhibitory apparatus was the cause of the apparent stimulation of the oculomotor center.

edge of the orbit and the eyeball was pulled forward. The ocular muscles were spread with a retractor and a short slit was made lengthwise in the sheath of the optic nerve, which was then doubly ligated and sectioned between the ligatures. After recovery (10 days to 2 weeks) it could be demonstrated that no appreciable damage had been done to the autonomic innervation. The eyes were tested before and after each experiment, and it was invariably found that both pupils contracted when light was allowed to fall on the right eye, and that no effect was obtained from the left, showing not only that the autonomic nerves were intact, but also that the left optic nerve had been successfully severed.

Ten milligrams of morphine sulfate per kilogram was then injected into one of the dogs, and, when the drug had taken effect, the right eye was covered for a few minutes. The diameter of the left pupil, as measured by holding a millimeter rule in front of the eye, was now 4.9 mm. On uncovering the right eye, the pupil size promptly decreased to 2.9 mm. Altering the amount of light falling on the blind eye had no effect on the pupil size. This was repeated with similar results in each of the five dogs operated on. It is therefore obvious that the size of the pupil in the morphinized animal does depend on the integrity of the optic nerve; Picard's conclusions in this respect are not confirmed. In the results and figures to follow, with the exception of the experiment on the human eye, all data were obtained from measurements and photographs of the reaction of the left eye (optic nerve cut) to light of varying intensities falling upon the retina of the right, or intact eye. Since both pupils were constricted to an almost exactly equal degree it seemed unnecessary to include the measurements from the control eye (with intact optic nerve).

Amount of light and degree of miosis. Three intensities of light were used: 5, 28 and 49.1 foot candles. The first was the usual room light; the second two were obtained from a standard giant ophthalmoscope held about 5 cm. from the cornea so that light would strike the retina approximately parallel to the normal visual axis. Measurements of the diameter of the pupil (usually in the transverse axis) were made either by holding a millimeter scale in front of the eye, or, for greater accuracy, from flash-bulb photographs. The time taken to secure a photograph in this way was so short that no change in pupil size occurred, and there was no significant difference in the results obtained by these methods. The broken lines in fig. 1 show the findings in dogs given 4.5 mg. of morphine sulfate per kgm. subcutaneously, the solid lines indicating the results in the absence of the drug. There is no question that the degree of miosis in both cases depends on the amount of light, and that with the same amount of light the pupil after morphine is almost invariably smaller than the corresponding control. The only exception to this (dog 3B) is probably to be explained by the effects of morphine wearing off, since these observations were made nearly three hours after the injection of the drug.

Another interesting feature is that in about half the controls the increase in degree of constriction with increase in light is greater between 28 and 49 foot candles than it is with weaker illumination, while after morphine the opposite

and Feilchenfeld (8). In their figure 1 we see that as the intensity of illumination on the normal eye is increased, pupillary constriction increases first slowly, then more rapidly, and finally more slowly again. Our controls correspond to the right half of their curves, while our morphine curves resemble very closely the half with higher illumination. Morphine, then, appears to produce results similar to those obtained with increased light; that is to say, its effect is to intensify the light reflex.

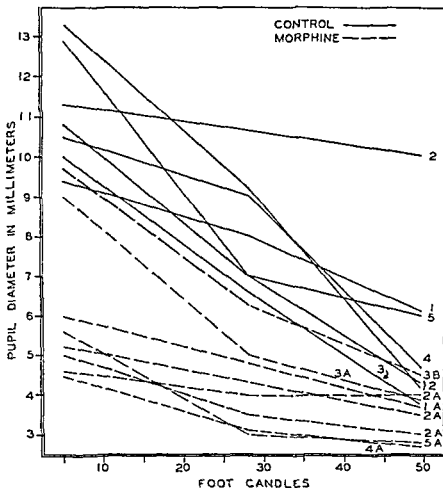


FIG. 1. EFFECT OF MORPHINE (4.5 MGM. PER KGM.) ON PUPIL DIAMETER OF 5 DOGS IN WHICH THE LEFT OPTIC NERVE HAD BEEN CUT

Curves obtained with the same dog are given the same number; the letter A denotes curves taken 1 to 1½ hours after subcutaneous administration of morphine; the letter B 2 hours, 55 minutes after.

Effect of morphine in complete darkness in dogs with the left optic nerve cut. If the degree of constriction of the pupil were determined solely by the light reflex, then morphine should have no effect on the pupillary size in complete darkness. This is rather unlikely, and we have found, using flash-bulb photographs, that morphine will constrict the pupil somewhat when no light is falling on the retina. This indicates that other factors are at work without, however, showing what they are.

The human eye. Since the "pin-point" pupil has usually been observed in the human eye, and the results given above were obtained in the dog, it seemed

possible that there might be a species difference. Accordingly one subject was given a dose of 16 mgm. of morphine sulfate intramuscularly following which the diameter of the pupil was measured by the flash-photograph technique in total darkness and in light of varying intensity. The results are shown in figure 2 and are exactly the same as those obtained in the dog: the size of the pupil in total darkness is somewhat decreased, and there is a further decrease as light of increasing intensity is allowed to fall on the retina.

The effect of prostigmine. Since prostigmine increases other effects of morphine (Slaughter and Munsell, 9), it seemed probable that it might increase the miosis also. This was found to be the case in the dog. Prostigmine alone, in doses up to 0.03 mgm. per kgm. injected subcutaneously or intravenously, produced no

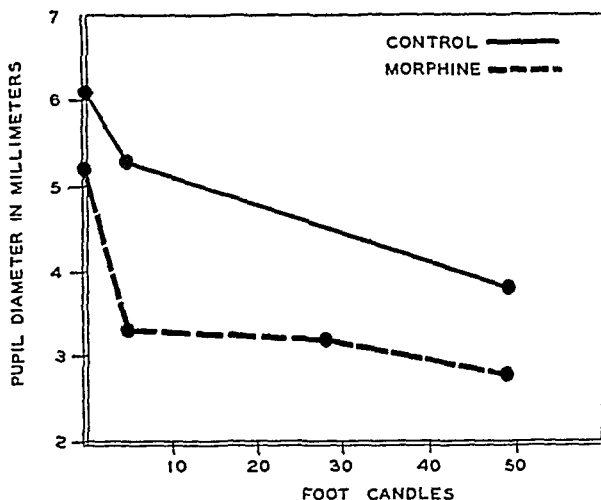


FIG. 2. EFFECT OF MORPHINE ON THE HUMAN EYE

The second curve was obtained 1 hour and 10 minutes after a subcutaneous dose of 16 mgm. of morphine.

significant change in pupillary diameter in dogs. Following control measurements in 5 dogs with the left optic nerve cut, morphine sulfate was administered subcutaneously (3.0 to 4.5 mgm. per kgm.) and about one and a half hours later measurements were repeated. Prostigmine (0.015 to 0.02 mgm. per kgm.) was then given subcutaneously and further measurements made. Reference to figures 3 and 4 will show that the morphine effect is increased by prostigmine. Curve D of figure 4 is anomalous in that on increasing the light from 28 to 49 foot candles no further increase in constriction occurred, although with morphine alone an increase did occur, so that at the higher illumination the pupil was slightly larger after prostigmine and morphine than after morphine alone. The reason for this exception is obscure.

It may be remarked incidentally that in several cases pronounced photophobia

light. No photophobia was observed following the injection of prostigmine in the unmorphinized animal.

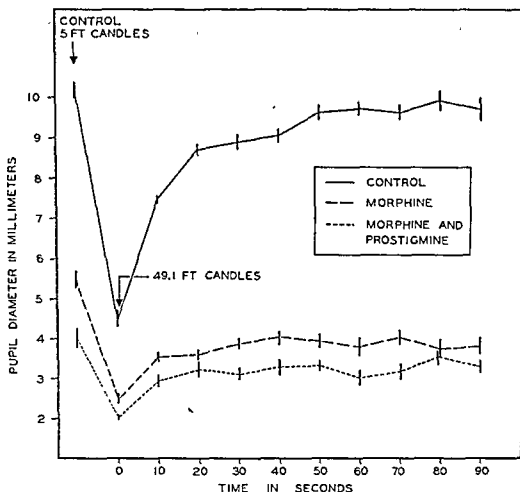


FIG. 3. EFFECT OF MORPHINE AND MORPHINE AND PROSTIGMINE ON RATE OF DILATATION OF THE PUPIL AFTER EXPOSURE TO STRONG LIGHT

below.

Number of measurements averaged for each point

	CONTROL BEFORE ILLUMI- NATION	ILLUMI- NATION LIGHT INTENSITY 49.1 FT. CANDLES	TIME INTERVAL IN SECONDS AFTER ILLUMINATION									
			10	20	30	40	50	60	70	80	90	
Control	41	41	110	106	82	70	57	52	55	61	58	
Morphine	27	27	66	48	45	32	33	28	32	27	24	
Morphine and prostigmine	16	16	42	35	25	23	19	21	19	20	16	

Rate of recovery from exposure to light. The rate of pupillary dilatation after exposure to strong light (49.1 foot candles) is shown in figure 3 both before and after morphine and after morphine and prostigmine. These are composite

curves of the 5 operated dogs, with the standard error of the mean indicated. It will be observed that in the controls recovery from the miosis is comparatively rapid at first, while the later portion of the recovery is very considerably slower, so slow in fact that recovery to the original control level was usually not quite

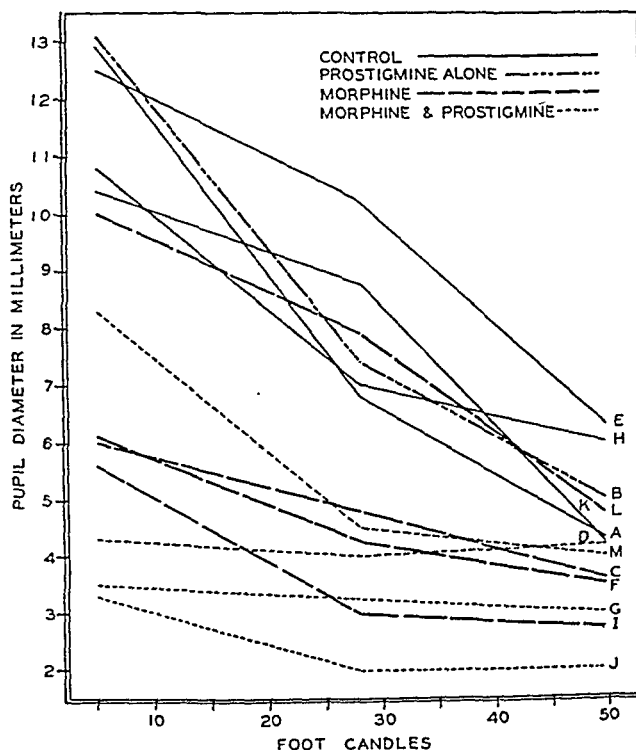


FIG. 4. EFFECT ON PUPIL DIAMETER OF PROSTIGMINE FOLLOWING MORPHINE
(SAME DOGS AS FIGURES 1 AND 2)

Dog no. 2. Curve A, control. Curve B, prostigmine alone 0.02 mgm. per kilo. Curve C, $1\frac{1}{2}$ hours after morphine 4.5 mgm. per kilo. Curve D, Prostigmine 0.02 mgm. per kilo immediately following curve C.

Dog no. 2. Curve E, control. Curve F, $1\frac{1}{2}$ hours after morphine 3.0 mgm. per kilo. Curve G, prostigmine 0.018 mgm. per kilo immediately following curve F.

Dog no. 4. Curve H, control. Curve I, $1\frac{1}{2}$ hours after morphine 4.5 mgm. per kilo. Curve J, prostigmine 0.015 mgm. per kilo immediately following curve I.

Dog no. 3. Curve K, control. Curve L, $1\frac{1}{2}$ hours after morphine 4.5 mgm. per kilo. Curve M, prostigmine 0.017 mgm. per kilo immediately following curve L.

complete during the interval in which measurements were made. The effect of morphine is to decrease greatly the rate of recovery in both its phases. Under these circumstances, prostigmine administered after morphine further increases the sensitivity to both room and bright light; there is, however, apparently no further alteration in the rate of recovery of the pupil in room light.

DISCUSSION. The finding that morphine miosis does depend very largely on the integrity of the optic nerve deprives the hypothesis of a direct central stimulation of its chief support. Moreover, this and the relationship between degree of miosis and retinal illumination are very difficult to explain on such a theory, and point rather to an increased sensitivity of the reflex arc to light as the cause of the greater part of the miosis. Breaking this arc anywhere would result in mydriasis, and it is probable that the results of Henderson and Graham (3) are to be accounted for in this way.

The delayed recovery from the miosis produced by light of an intensity of 49.1 foot candles, after the administration of morphine as compared with the unmorphinized animal, might be due to sympathetic action. There is, however, no other evidence of paralysis of sympathetic fibers, and it seems more probable that this phenomenon is somewhat analogous to the "after-discharge" of spinal reflexes in that oculomotor neurones may go on discharging for quite a long time after afferent stimulation has ceased, so long in fact that the delay has an apparently cumulative character. Another possibility is that the transmission of impulses from nerve to muscle is altered by morphine so that the muscular response is increased in both intensity and duration, or there might be a combination of both of these mechanisms. In any case, the increased sensitivity suggested above must be regarded as a change producing not only an increased degree of response, but also an increased duration, at least on the part of some of the fibers.

Increased sensitivity to the light reflex is, however, obviously not the whole explanation, since there is some miosis in complete darkness. Our experiments offer no explanation of this residual miosis. The hypothesis of direct stimulation, although to a lesser degree, may be retained to account for it, but, on the other hand, it is possible to argue by analogy that since one reflex is exaggerated, other constrictor reflexes may be similarly affected. In the third place a peripheral effect on the iris is not excluded, for Poos (10) found that morphine produced a contraction of the isolated sphincter just as eserine did. Increased sensitivity, then, will explain part of the phenomenon, and may or may not explain all of it.

As to the mechanism of the increased sensitivity, the potentiating effect of prostigmine is suggestive, but not, however, conclusive. Morphine, as well as prostigmine and eserine, is known to inhibit cholinesterase (11, 12, 13) and indeed other of its effects have been thought to be due to this mechanism (9). It is also probable that there are several cholinergic transmissions involved in the reflex arc of the light reflex. Not only is there a certain amount of evidence for this within the central nervous system (cf. Fulton, 14), but Velhagen (15, 16) found a substance which was probably acetylcholine in the aqueous humor, iris, ciliary body and retina. Moreover, Chang and Gaddum (17) found some evidence for the formation of choline esters in the eye on stimulation of the oculomotor nerve, and, finally, Engelhart (18) demonstrated that on illumination of the eye the amount of acetylcholine is considerably increased not only in the retina, but also in the ciliary ganglion and iris. There is, therefore, the possibility of inhibition of cholinesterase in this particular reflex arc. Such an inhibi-

tion would probably result in increased responses, both in intensity and duration, and it would be increased by prostigmine. The whole question is, however, complicated, and much more work is necessary to elucidate it completely.

SUMMARY

1. Morphine miosis in the dog is to a large extent dependent on the integrity of the optic nerve.
2. The degree of miosis in both dog and man depends very largely, but not altogether, on the amount of light falling on the eye, so that the effect of morphine appears to be mainly an exaggeration of the light reflex.
3. These effects of morphine are potentiated by doses of prostigmine which are in themselves ineffective.
4. The rate of recovery of the pupil from a brief exposure to a bright light is greatly delayed by morphine but does not appear to be further delayed by prostigmine.
5. The significance of these findings is discussed.

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SOLUBILITY STUDIES AND THE ORAL ADMINISTRATION OF SODIUM SULFAPYRIDINE^{1,2}

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Oral sodium sulfapyridine and sodium sulfathiazole have been studied in both animals and man. Marshall *et al.* (1, 2) utilized soluble sodium sulfapyridine for toxicity evaluation in animals. Gastro-intestinal and other lesions with this material were noted by Molitor and Robinson on oral feeding in rats (3). Sadusk *et al.* (4) noted a rapid rise in blood levels of free drug with oral sodium sulfathiazole, and Ratish and co-workers (5) first suggested the oral use of sodium sulfapyridine in therapeutics on the basis of human data indicating almost immediate absorption. Barlow and Climenko (6) pointed out that in the oral use of sodium sulfonamides, "the absorption of such a salt from the gastro-intestinal tract should be primarily a function of the quantity of free acid in the stomach and the emptying time of the stomach." Rapid absorption with these sodium salts was noted in man, with still more rapidly rising and higher blood levels in monkeys (6). Conclusions similar to those of Barlow and Climenko were reported by Strauss *et al.* (7). Still more recently, sodium sulfonamides have been recommended in therapy by Spink (8) and by Cook (9).

Differences between human and animal gastro-intestinal physiology and the lack of controlled experimentation in man have led us to reexamine the problem of oral administration of sodium sulfonamides.

STUDIES WITH GASTRIC JUICE. The solubility of the sodium sulfonamides depends upon the maintenance of a relatively high pH. Potentiometric titration curves of Lott and Bergeim (10) for acidification of sodium sulfathiazole and sodium sulfapyridine solutions show beginning precipitation of free sulfonamide at pH 8.2 and 9.6 and complete precipitation at pH 6.5 and 8.8, respectively. It was difficult to understand why gastric juice of dogs did not have sufficient buffering capacity to liberate free sulfapyridine completely from its sodium salt, as noted by Marshall and Litchfield (2). If this were also true in man, it would mean the rapid achievement of high concentrations of soluble absorbable sulfonamides within the gastro-intestinal tract. Therefore, we have constructed electrometric titration curves of sodium sulfapyridine with human gastric juice to determine its buffering capacity.

Method. Pooled, filtered, human gastric juice of pH 1.48 was used. A series of solutions of 5 per cent sodium sulfapyridine of 20 cc. volume were titrated with 5 cc. increments of gastric juice. Precipitated free sulfapyridine was removed

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TABLE 1

SAMPLE	VOLUME OF 5 PER CENT SODIUM SULFAPYRIDINE	VOLUME OF GASTRIC JUICE (pH 1.48)	pH FILTRATE	SOLUTION OF SULFAPYRIDINE IN FILTRATE
	cc.	cc.		per cent
1	20	5	10.0	3.4
2	20	10	9.85	2.7
3	20	15	9.71	2.15
4	20	20	9.63	1.6
5	20	25	9.45	1.28
6	20	30	9.30	.91
7	20	35	9.11	.73
8	20	40	8.83	.485
9	20	45	8.47	.188
10	20	50	7.35	.104
11	20	55	4.57	.095
12	20	60	3.35	.113
13	20	65	2.85	.134
14	20	70	2.60	.137

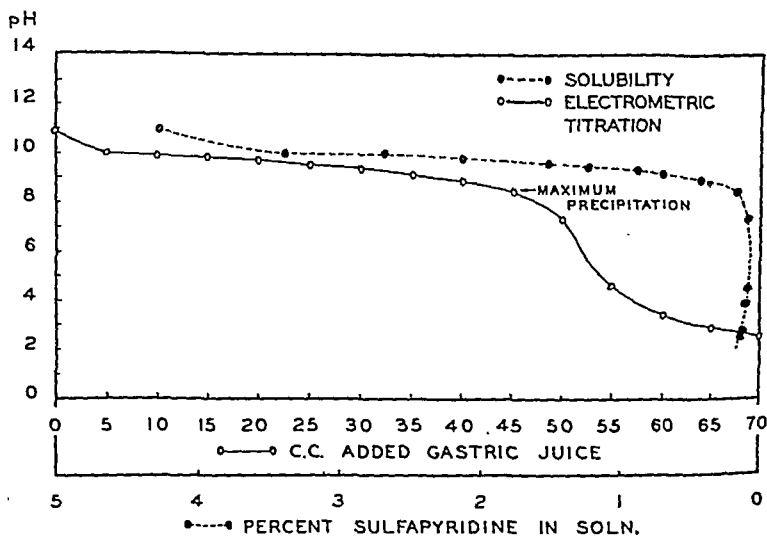


FIG. 1. ELECTROMETRIC TITRATION CURVE OF 5 PER CENT SODIUM SULFAPYRIDINE SOLUTION WITH POOLED HUMAN GASTRIC JUICE OF pH 1.48

Each point represents individual titration samples. The pH is plotted on ordinates and cubic centimeters of added gastric juice plotted on the abscissae. The superimposed sulfapyridine solubility curve shows the solubility of the drug plotted on the abscissae at the pH indicated on the ordinates.

and the pH of the solution determined by the Coleman glass electrode. The sulfapyridine concentration of the filtrate was determined by the method of Marshall and Bratton (22) using the Evelyn colorimeter.

Results. (See fig. 1, table 1.) Precipitation of free sulfapyridine occurs with a trace of added gastric juice. The constant addition of gastric juice to sodium sulfapyridine solutions steadily increases precipitation with small changes in pH until maximum precipitation has occurred (pH 8.5-8.8); at that moment a sudden pH change is noted. The graph for this titration resembles very closely that of Lott and Bergeim for the titration with 0.1 N HCl.

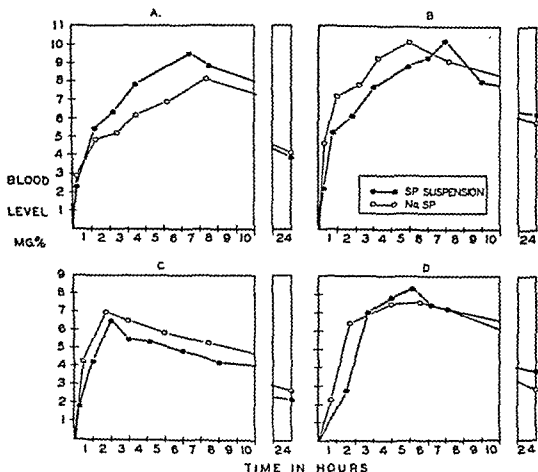


FIG. 2. COMPARATIVE ABSORPTION CURVES (FREE BLOOD SULFAPYRIDINE) AFTER ORAL SULFAPYRIDINE (4.0 G.) AND SODIUM SULFAPYRIDINE (4.64 G.) IN FOUR NORMAL PATIENTS

Time postprandial: A = 2½°; B = 2½°; C = 3½°; D = 1½°. The legend for figures 2 and 3 is indicated in this figure. Blood level in milligrams per cent plotted on ordinates and time hours on the abscissae.

Plotted on figure 1 is the curve of drug concentration at varying pH. Concentration of sulfapyridine decreases quite rapidly with little or no change in alkalinity until pH 8.5 and a concentration of 180 mgm. per cent have been reached. A sharp drop in pH with no appreciable change in drug solubility is noted below pH 8.5. It is a corollary of these observations that for sodium sulfapyridine to exist in the gastro-intestinal tract we must assume an environmental pH of 8.5 or higher. From the data of Lott and Bergeim, a pH of 6.5 is the critical level for sodium sulfathiazole.

These data indicate that acid human gastric juice quantitatively precipitates free sulfapyridine from a solution of its sodium salt.

ABSORPTION STUDIES ON MAN. Published observations on oral sodium sulfonamides in man, were made under conditions that were not always standard or constant. Few attempts have been made to follow pH changes of stomach contents and no correlation between absorption data and gastric pH changes has been presented. With these objectives, the following experiments were performed.

Method. Convalescent patients were given equimolar quantities of sulfapyridine and sodium sulfapyridine; the doses were 4.0 and 4.64 G., respectively,

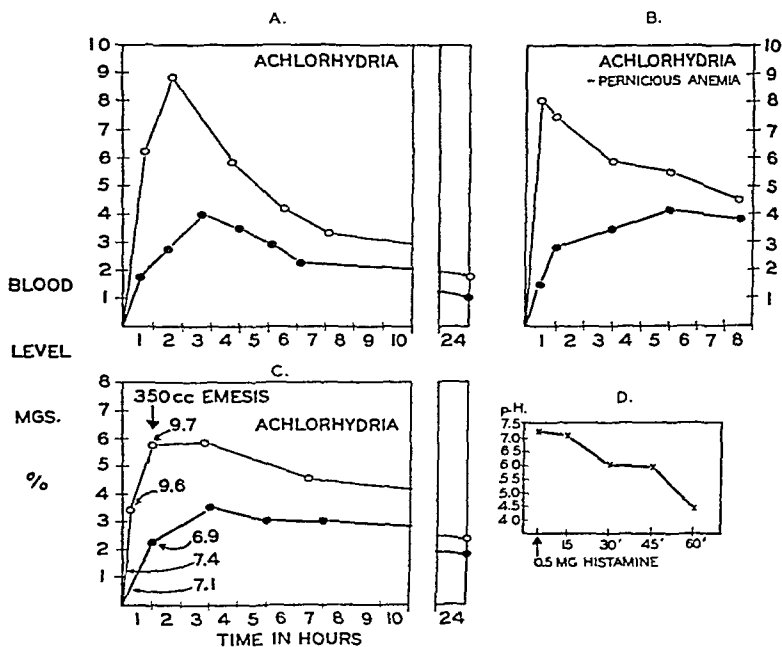


FIG. 3. COMPARATIVE BLOOD ABSORPTION CURVES AFTER ORAL SULFAPYRIDINE AND SODIUM SULFAPYRIDINE IN PATIENTS WITH HISTAMINE PROVEN ANACIDITY

Patient B received 3.0 G. and 3.48 G. of sulfapyridine and sodium salt, respectively. Patients A and C received 4.0 and 4.64 G., respectively. The small figures of figure C refer to pH of the aspirated gastric juice at the moment the blood sample was taken.

except in two patients in whom the dosages were 3.0 and 3.48 G. (figs. 3 B and 4 D). The vehicle for administration was the same in any one patient: 5 per cent flavored acacia or water. Sulfapyridine was mixed with rapid stirring until a homogenous suspension was formed. Sodium sulfapyridine readily dissolved in either water or acacia. The drugs were given with 180 cc. of vehicle and followed by 180 cc. of water. In a few patients commercial tablets of sulfapyridine were given with a similar quantity of fluid. Over and above the dietary water, the daily fluid intake was constant. Both drugs were given the same time of day. Observations of figure 2 were recorded $1\frac{1}{2}$ to $3\frac{1}{2}$ hours after breakfast; others were

studied in the fasting state. Blood samples were taken before drug administration, at intervals during the first 8 hours, and again after 24 hours. Seventy-two hours were allowed for clearance of the blood but a correction was introduced when necessary. Free sulfapyridine alone was determined, inasmuch as other factors have been adequately studied. Where indicated, a Levin tube was passed

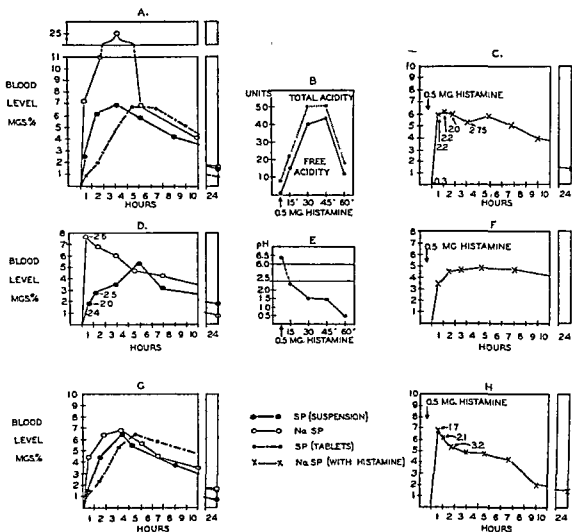


FIG. 4. COMPARATIVE ABSORPTION CURVES (FREE BLOOD SULFAPYRIDINE) AFTER ORAL SULFAPYRIDINE AS TABLETS AND SUSPENSION IN WATER, ORAL SODIUM SULFAPYRIDINE, AND ORAL SODIUM SULFAPYRIDINE PRECEDED BY 0.5 MG. HISTAMINE ON PATIENT WITHOUT FREE FASTING ACID BUT WITH NORMAL RESPONSE TO HISTAMINE.

Each group of figures ABC, DEF, and GH is from an individual patient. Histamine

into the stomach and the pH of aspirated samples was determined. Control gastric analyses (histamine) are also noted.

Results. The results of these observations are recorded in figures 2, 3, and 4. The time-blood concentration curves of free sulfapyridine of four patients are seen in figure 2. The blood level in milligrams per cent is plotted on ordinates and the time in hours on the abscissae.

A slightly increased rate of absorption⁴ with sodium sulfapyridine is quite clearly seen in some patients (fig. 2 B and C). In the first half hour the blood levels average 4.5 mgs. per cent with the sodium salt as compared with 2.0 mgm. per cent with the free acid. In figure 2 A the early absorption rate is slightly greater with the sodium salt, but the peak level is slightly increased after sulfapyridine. Differences of this degree are seen with the same drug (14). In all four subjects, after the initial rapid absorption phase, both curves for each subject are surprisingly similar. This would indicate similar treatment of these materials *in vivo* (4-6). A general finding in these observations is that the maximal free drug level is the same whether the free drug or its sodium salt is given. Differences that exist are in the time necessary to reach this peak. This would seem to indicate adequate buffering capacity of human gastric juice *in vivo*.

Achlorhydria. Marked differences in the time-blood concentration curves of sulfapyridine and sodium sulfapyridine were not infrequently encountered (fig. 3 ABC). The subjects of figure 3 were all proven achlorhydric. Comparative increase in blood levels are 1.8 to 6.1 and 1.5 to 8.1 mgm. per cent in less than an hour, for sulfapyridine and sodium sulfapyridine, in patients A and B, respectively. The differences in the curves of figure 3 C are less striking due to repeated emesis of the sodium salt. In this patient, the rise in pH of stomach contents from 7.4 to 9.7 one hour after sodium sulfapyridine indicates that in the absence of an acid gastric juice the buffering capacity of stomach contents is not present.

These observations on achlorhydria indicate that rapid high absorptive rate with oral sodium sulfapyridine may be associated with incomplete or absent buffering by gastric juice.

Fasting anacidity. Not all patients with differences in absorption rates evidenced a complete achlorhydria (fig. 4 AD). The patient of figure 4 A proved quite informative. Administration of sulfapyridine resulted in a maximal peak of 7.0 mgm. per cent. Oral administration of sodium sulfapyridine was followed by a very rapidly increasing blood level of the drug to 7.2 mgm. per cent in 25 minutes, and 25 mgm. per cent in 3¼ hours. No free fasting hydrochloric acid with a normal response to histamine was noted on gastric analysis. Oral sodium sulfapyridine was then repeated, preceded by 10 minutes with 0.5 mgm. histamine (fig. 4 C). A rapid rise in blood level (6.8 mgm. per cent) was again elicited but succeeding blood levels did not rise above 7.2 mgm. per cent. Gastric contents showed no free-fasting acid but further samples contained free acid.

A similar response was noted in the subject of figure 4 D. Fasting gastric juice was pH 3.75. This is the limiting value of free hydrochloric acid (11, 12). The blood level 30 minutes after sodium sulfapyridine was 7.6 mgm. per cent as compared to 1.5 with the free acid. Readministration of the sodium salt one-half hour after 0.5 mgm. histamine resulted in a slower absorption and lower peak

⁴ It should be pointed out that "absorption" as used in this sense is strictly not absorption, but a measure of the relative factors of absorption, distribution, and excretion. From the observations of others on the rate of excretion in the first few hours, and similar conditions of administration of both drugs, the use of "absorption" with this meaning is possibly justified.

(fig. 4 F). This latter curve is similar to the one obtained with sulfapyridine alone (fig. 4 D).

The histamine effect on the absorption of oral sodium sulfapyridine in a normal subject is seen in figure 4 GH. An increased absorption rate was undoubtedly due to an increased motor activity of the stomach from the histamine.

Note on particulate size and dispersion. In a few patients, sulfapyridine (as free acid) was given in two forms to determine the effect of dispersion and particulate size upon absorption in man: suspension in water and in half-gram tablets (fig. 4 A and G). More rapid absorption was noted when the drug was given as a suspension even though total fluid intake was the same with both procedures.

DISCUSSION. Animal and human studies with sodium sulfonamides have indicated increased gastro-intestinal absorption over the acid sulfonamides. Anacidity in animals is not uncommon, however, and has not been previously considered. Manville (13) found that the pH of a normal rat stomach was about 3.4. This is about the minimal accepted level of free hydrochloric acid (11, 12). In fasted white rats, we have found stomach pH values of 3.7 to 5.4 (14). The experimental data of Barlow and Climenko (6) on rapid absorption in monkeys should be considered in the light of the observations of Schnedorf and Ivy (15) who found 6 of 12 Rhesus monkeys achlorhydric to 0.5 mgm. histamine. None of these 12 animals secreted free acid in the fasting state or in response to alcohol or to a test meal. It is understandable why fasted animals in whom gastric acidity would be low were found to be the best test object in the pharmacologic evaluation of these drugs (2).

In view of these findings in animals it is interesting that patients showing more rapid and complete absorption with sodium sulfapyridine were either achlorhydric or had no free acid when fasting. The modification of the absorption curve of sodium sulfapyridine by histamine in patients without fasting acid is confirmatory evidence that incomplete secretory activity had been previously in effect. A logical inference from these observations is that the more normal the gastro-intestinal tract the less the difference in absorption between oral free acid sulfonamide and its sodium salt.

The varied observations in the different gastro-intestinal states is more clearly understood when one considers the titration curves of the sodium sulfapyridine solutions with normal human acid gastric juice. Little alkaline salt would be present if gastric function were normal. More recent studies by Eyerly (16) using the glass electrode *in situ* demonstrate that in the stomach, pylorus, and distal duodenum the pH remains below pH 7.0 in spite of intensive antacid therapy. This illustrates a resistance to abnormal pH within the gastro-intestinal tract and the difficulty of alkalization of the normal tract by sodium sulfonamides. Such theoretical considerations are borne out by our experimental data.

Local toxic effects of these oral alkaline solutions have been pointed out in animals (3) but denied in man (5). Gastric acidity must have an important rôle in protection against these changes. It remains to be shown that such therapy is innocuous in achlorhydric humans. Gastric anacidity in man is not uncommon.

It is seen in at least 1 to 2 per cent of apparently normal individuals (17) and unpredictable spontaneous achlorhydria is not uncommon (18). Anacidity increases in old age and in the diseased states (19). Possibly related to human toxicity is the immediate nausea and vomiting that are a common accompaniment of oral sodium sulfonamides (4, 6, 14).

Marshall and Litchfield (2) noted that in dogs the physical state of administered sulfapyridine was of importance in the rate of absorption. We have observed similar results in man. This was previously noted by Mayer (20) using benzyl-sulfanilamide. We are at a loss to explain lack of confirmation of these observations by Bullowa (21). For rapid and maximal absorption it would appear desirable to administer these drugs as the free acid suspended in a considerable volume of fluid.

It is believed that with some modification of actual pH values, these same general conclusions can be applied to sodium sulfathiazole and sodium sulfadiazine.

CONCLUSIONS

1. Normal human gastric juice is an excellent buffer against sodium sulfonamides. The electrometric titration curves of sodium sulfapyridine solution with gastric juice closely simulates that obtained with 0.1 N HCl.

2. In normal individuals, the time necessary to reach a maximal blood level is somewhat less with oral sodium sulfapyridine than with the free acid. Maximal levels do not differ with sodium salt and free acid administered orally.

3. Absorption of sulfonamide is much more rapid in achlorhydria or in those with no free fasting hydrochloric acid when the sodium salt is administered. Previous administration of histamine will slow absorption rate when no hydrochloric acid is present in only the fasting specimen.

4. The particulate size and dispersion of the administered material may be of importance in the rapidity of absorption.

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It is seen in at least 1 to 2 per cent of apparently normal individuals (17) and unpredictable spontaneous achlorhydria is not uncommon (18). Anacidity increases in old age and in the diseased states (19). Possibly related to human toxicity is the immediate nausea and vomiting that are a common accompaniment of oral sodium sulfonamides (4, 6, 14).

Marshall and Litchfield (2) noted that in dogs the physical state of administered sulfapyridine was of importance in the rate of absorption. We have observed similar results in man. This was previously noted by Mayer (20) using benzyl-sulfanilamide. We are at a loss to explain lack of confirmation of these observations by Bullova (21). For rapid and maximal absorption it would appear desirable to administer these drugs as the free acid suspended in a considerable volume of fluid.

It is believed that with some modification of actual pH values, these same general conclusions can be applied to sodium sulfathiazole and sodium sulfadiazine.

CONCLUSIONS

1. Normal human gastric juice is an excellent buffer against sodium sulfonamides. The electrometric titration curves of sodium sulfapyridine solution with gastric juice closely simulates that obtained with 0.1 N HCl.

2. In normal individuals, the time necessary to reach a maximal blood level is somewhat less with oral sodium sulfapyridine than with the free acid. Maximal levels do not differ with sodium salt and free acid administered orally.

3. Absorption of sulfonamide is much more rapid in achlorhydria or in those with no free fasting hydrochloric acid when the sodium salt is administered. Previous administration of histamine will slow absorption rate when no hydrochloric acid is present in only the fasting specimen.

4. The particulate size and dispersion of the administered material may be of importance in the rapidity of absorption.

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guinea pigs of non-albino strains, about 6 or 8 weeks in age, maintained on the "Rockland" Guinea Pig" ration. To prevent coagulation the blood was drawn with a syringe containing 0.1 cc. of 0.1 M sodium oxalate per cc. of blood to be removed.

The blood was centrifuged, the plasma was carefully separated, and diluted with twice its volume of calcium-free Ringer's solution. The composition of the latter was identical with the Ringer's solution used to dilute the liver extract, except that CaCl_2 was not added.

Before proceeding further it was found advisable to test the coagulability of this diluted plasma, as excessive hemolysis or slight clot formation before centrifugation will prevent coagulation later. To 0.75 cc. of normal Ringer's solution was added 0.25 cc. of the diluted plasma. Coagulation usually occurred within 10 minutes.

If coagulation was satisfactory the marrow was prepared. Both femurs were removed aseptically and the bone was split longitudinally, whereupon the marrow could be removed almost intact. The marrow was then cut with a razor blade into pieces 0.5 mm. to 1.0 mm. in diameter.

A volume of 0.75 cc. of each dilution of the liver extract to be studied was placed in a separate Stender dish measuring 36 mm. by 24 mm. In addition two dishes were prepared each containing 0.75 cc. of normal Ringer's solution to serve as controls. Six pieces of marrow were placed in each dish, and these were followed by the addition of 0.25 cc. of the diluted plasma. After gentle swirling, the bits of marrow were spaced evenly and the dishes were set aside until the mixture had coagulated. The calcium in normal Ringer's solution was sufficient to precipitate the oxalate and to provide an excess of calcium for coagulation.

When the plasma had set firmly, the dishes were incubated at 37.5° for 15 hours. During this time cells from the marrow moved out into the surrounding coagulated plasma and formed a definite zone of migration. The pieces of marrow with their surrounding migration fields were projected onto paper, and the outlines of the marrow and migration field were traced. A Spencer Delineascope with minor changes proved suitable for projection. Occasionally, difficulty was encountered in determining the exact limits of migration, but usually the limits were sharply defined. The observer who traced the areas did so without knowing the dilution value of liver extract involved.

The areas of the marrow and its migration field, which values were to be used in determining the degree of migration, were readily determined by means of a planimeter. The coefficient of migration for each piece of marrow was calculated by the formula

$$M = \frac{O_2 - O_1}{O_1}$$

where O_2 and O_1 are the areas of migration and of the marrow respectively. (After Gaillard *et al.* (15).)

In order to compare the results from various determinations more directly, we expressed the average M values for each dish as percentages of the average of the control dishes. These percental migration coefficients (P) were then plotted against the dilutions of liver extract employed, and a peak similar to that described by Gaillard and co-workers (15) was observed. The peak migration was more than 30 per cent greater than the control in every case. There seems to be a tendency to obtain greater maximal P values when working in the higher dilution ranges than in the lower ranges. The exact explanation of this is not clear.

RESULTS. The protocol of a typical determination is given in table 1, and the resulting curve is plotted in figure 1. In this instance a parenteral liver extract², rated at 5 U.S.P. units per cc., yielded a maximal migration coefficient at a dilution of 36,000 times. The dilution value of 36,000 was therefore considered equivalent to a potency of 5 U.S.P. units per cc. in this case.

² Abbott's Injectable Liver Extract, 5 U.S.P. units per cc.

TABLE 1

Typical determination of the migration of cells from bone marrow under the influence of liver extract

The method for making the dilutions is described in the text, as is the procedure for calculation of the migration coefficient, M , and the percental migration coefficient, P . The standard deviation from the mean, $S.D.$, the standard error of the mean, $S.E.$, and the significance value, t , are given in columns 10, 11, and 12. The value of P at the dilution 1 to 32,000 is significantly high, but as a general rule the highest P value was designated as the peak and in this case the peak was considered to be at the dilution 1 to 36,000 (see figure 1). The blanks in the table indicate pieces of marrow whose migration fields were difficult to trace owing to imperfections in the dishes, etc.

DISH NUM- BER	LIVER EXTRACT DILUTION	MIGRATION COEFFICIENT FOR EACH OF 6 MARROW SLICES IN EACH DISH						AVER- AGE \bar{M}	$S.D.$	$S.E.$	t	P
		1	2	3	4	5	6					
1	Control	17.5	19.1	26.5	17.8			20.1	3.4	1.08		100
2	Control	21.5	14.2	22.8	21.7	18.2	21.7					
3	16,000	18.0	16.6	16.2	12.7	19.1	19.5	17.0	2.5	1.02	-1.95	85
4	20,000	19.7	19.1	25.5	25.4	26.3		23.2	3.5	1.56	+1.68	115
5	24,000	21.9	19.1	25.8	19.2	15.8	16.6	19.7	3.7	1.51	-0.25	98
6	28,000	23.6	18.1	23.6	26.9	19.6	18.0	21.6	3.6	1.47	+0.82	107
7	32,000	26.9	26.8	23.0	28.8	28.6		26.8	2.3	1.03	+4.50	133
8	36,000	26.1	34.6	21.9	26.8	27.4		27.4	4.6	2.06	+3.14	137
9	40,000	23.2	14.1	15.7	21.4	16.6	17.0	18.0	3.5	1.43	-1.73	90
10	44,000	15.1	23.0	18.4	16.4	25.1	18.4	19.8	3.9	1.59	-0.16	99
11	48,000	21.7	24.3	20.0	20.6	31.0	17.6	20.9	5.0	2.04	+0.35	104
12	52,000	20.6	18.3	20.5	22.0	17.1	25.7	20.7	3.0	1.22	+0.37	103
13	56,000	19.7	22.3	17.6	21.6	19.9	18.5	19.9	1.5	0.61	-0.16	99
14	60,000	16.8	17.3	15.6	21.4	16.7	22.6	18.3	2.9	1.18	-1.75	91
15	64,000	19.4	17.5	17.9	24.3	24.7	25.7	21.6	3.7	1.51	+0.81	107

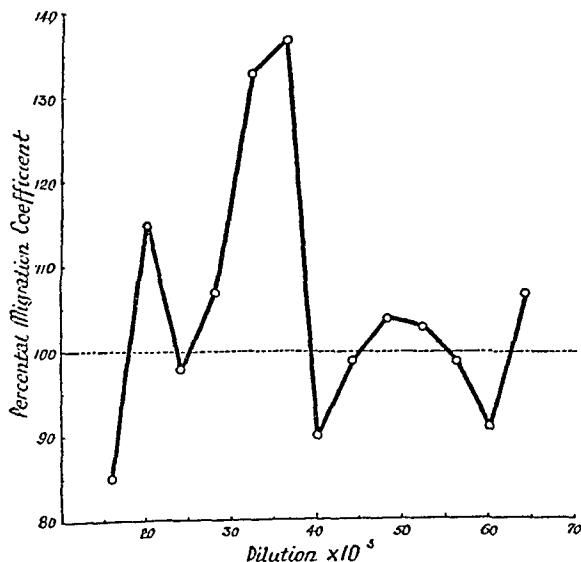


FIG. 1. A TYPICAL CURVE SHOWING THE EFFECT OF VARIOUS DILUTIONS OF LIVER EXTRACT ON THE CELLULAR MIGRATION FROM ISOLATED BONE MARROW

In figure 2 are plotted the results obtained from five commercial preparations bought on the open market, and to which had been assigned U.S.P. unit ratings of various values. Each was run in duplicate using the marrow from separate guinea pigs. Plotted on the ordinate are the U.S.P. units contained in each sample, and on the abscissa the dilution at which maximal migration occurred

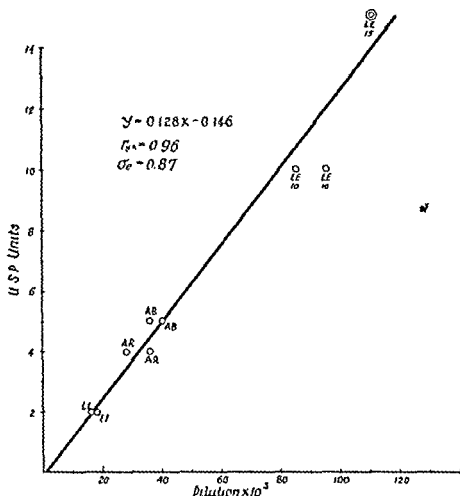


FIG. 2. RELATIONSHIP BETWEEN THE POSITION OF THE OPTIMAL DILUTION FOR MIGRATION, AND THE U.S.P. UNITAGE OF SEVERAL COMMERCIAL LIVER CONCENTRATES

LI represents "Lille's Solution Liver Extract" (U.S.P. Unitage 15)
 AR represents "Abbo"
 AB represents "Solut"
 LE 10 represents "Lille's Solution Liver Extract" (U.S.P. Unitage 10)
 LE 15 represents "Lille's Solution Liver Extract" (U.S.P. Unitage 15)
 The line is a line of best fit.

with the corresponding sample. The best straight line was determined by the method of least squares. The figure shows good linear correlation between these values.

DISCUSSION. Two regions of maximal migration were described by Gaillard, *et al.* (15) for a given liver concentrate, one at approximately 1 to 100 dilution and the other at approximately 1 to 10,000 dilution. They used the former in their work, whereas, we employed the latter in order to use liver concentrates without extracting the preservatives.

Gaillard and co-workers (15) could not demonstrate a statistically significant

difference between the migration coefficient obtained at the active dilutions and the migration coefficient at other dilutions. In table 1 are listed for each dish the standard deviation from the mean, the standard error of the mean, and the t value. The values at the two peak dilutions, 32,000 and 36,000, exhibit a significant difference from the controls by this method, the probability of their coming from a homogeneous parent being less than 0.01. Similar results were obtained from the other determinations.

Occasional experiments were made in which no clear-cut peak of migration occurred. However, on repeating the run, a typical response was obtained. We believe these aberrant results to be due to faults in technic rather than to a fundamental fault in the method, although we are not able to explain them fully at present.

Figure 2 represents the calibration curve for application of the method to the determination of expected clinical potency of unknown liver concentrates. The accuracy of the method in our hands agrees with that claimed by Gaillard, Overbeek, and Yam¹ (15).

From these results it would appear that the method may be used for the determination of the anti-pernicious anemia activity of an unknown liver concentrate. There are however, several aspects of the method which still require confirmation. Gaillard, Overbeek, and Yam (15) have presented evidence indicating a qualitative correlation between the clinical activity of an unknown extract and its activity on bone marrow *in vitro*. The fact that our points (fig. 2) exhibit practically a straight line relationship is, of course, further evidence of such correlation.³ They have also tested (15) the specificity to a limited extent. We have found that destruction of the clinical activity of a potent liver extract (either boiling for 1 hour with 2% sulphuric acid or racemization by exposing to one-half normal sodium hydroxide at room temperature for 48 hours and then neutralizing) will prevent the occurrence of the expected peak in an experiment using such an inactivated extract.

SUMMARY

The *in vitro* bone marrow assay method for anti-pernicious anemia preparations has been studied. A curve has been constructed showing the relationship between the dilution of liver concentrates at which maximal migration of cells from bone marrow occurred, and the U.S.P. potency of these concentrates. It would appear that this curve may be used for determination of the expected clinical potency of unknown liver extracts.

We are indebted to Dr. C. R. Spealman for the statistical analyses in this work, and to Dr. W. A. Peabody for his constructive criticisms and advice.

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THE PHARMACOLOGICAL BEHAVIOR OF THE INTRAOCULAR MUSCLES

IV. THE ACTION OF STRYCHNINE ON THE DILATOR AND SPHINCTER IRIDIS

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In the course of pharmacological experiments on excised strips of rabbit dilator and sphincter iridis, a hitherto unreported response to strychnine salts was observed (1).

The smooth muscles of the iris exhibit many peculiarities. Both the dilator and the sphincter differentiate from ectoderm, the sphincter more completely than the dilator. Ganglion cells, probably of sympathetic nature, are scattered throughout the iris. The neuromuscular complex of the mammalian iris, though hardly "phylogenetically primitive" as Boeke (2) suggests, is still "primitive" in the sense that the muscles are interblended and incompletely differentiated from their epithelial anlagen, and the nervous tissue is largely plexoid rather than synaptic. The sphincter action can be depressed by sympathicomimetic agents (3, 4, 5, 6), and by inhibiting impulses transmitted by the cervical sympathetic nerve (7). It seemed to be of interest to study the action of strychnine combined with adrenergic and cholinergic agents.

MATERIAL AND METHODS. Albino rabbits were used. The method of preparing the strips of iris tissue was that suggested by Poos (4) and adopted by Heath and Geiter (8). The holding and recording apparatus was that described by Heath and Sachs (1). The temperature of the Sollmann-Rademaeker solution¹ in which the muscle was submerged was $33 \pm 0.2^\circ\text{C}$., and the pH was 7.5 ± 0.1 . The method of application of the drugs, by means of a special syringe, has been described by Sachs and Heath (9). The volume of fluid in the muscle chamber was 200 cc. in most of the experiments, and 100 cc. in a few. The load on the muscle was kept constant at 20 mgm. The strychnine was used either as the sulfate or the chloride, both of which behaved alike.

A contraction was first produced in the dilator with adrenalin (chloride), and in the sphincter with either acetylcholine (chloride) or eserine (salicylate). Strychnine was then added, and its effect on the state of contraction of the muscle recorded. The concentration of the primary drugs was varied, but that of the strychnine was kept constant at 1:40,000 or 1:50,000.

RESULTS. *The normal sphincter and dilator.* Strychnine was tried alone on 13 sphincter strips and on 6 dilator preparations. Neither contraction nor relaxation was noted in any instance. Hence it may be concluded that strychnine, when administered alone, is without effect upon normal iris muscles.

The adrenalin-strychnine-dilator system. Fourteen dilator strips were used,

¹ The composition of this solution: Sodium chloride, 0.9 gram; potassium chloride, 0.04 gram; calcium chloride, 0.024 gram; sodium bicarbonate, 0.03 gram; dextrose, 0.1 gram; distilled water, 100 cc.

some of these more than once (by washing between the tests), so that in all 23 experiments were made. The initial contraction was produced with the adrenalin in either 1:1,000,000 or 1:2,000,000 concentration. In 22 of these experiments a subsequent application of strychnine produced a relaxation—which did not, however, reestablish the original length of the muscle. A sample experiment is illustrated in graph A of figure 1.

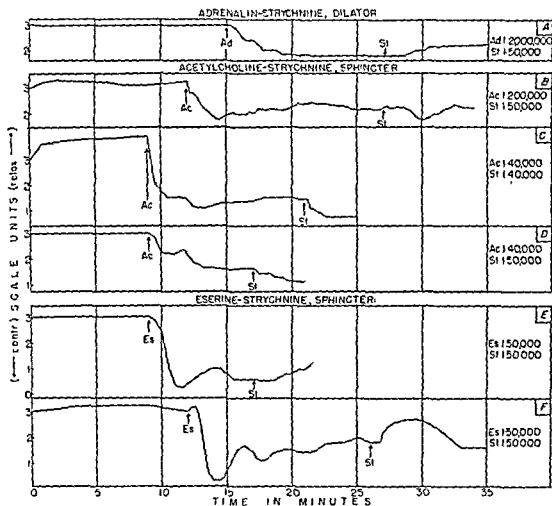


FIG. 1. SAMPLE TRACINGS OBTAINED FROM ALBINO RABBIT IRIS MUSCLE STRIPS UNDER DRUG STIMULATION
Explanation in text

In two animals, the left superior cervical ganglion was excised. After 16 and 18 days, respectively, tests were made (as above) upon three dilator strips from the denervated (homolateral) eye of each rabbit. The denervated eye being sensitized, the concentrations of adrenalin were low: 1:2,000,000–1:200,000,000. One experiment was doubtful, but in the other five the strychnine caused distinct relaxation comparable to that shown in graph A. Sympathetic ganglionectomy therefore does not abolish the action of strychnine.

The acetylcholine-strychnine-sphincter system. Thirteen experiments (on 10 strips) were made with acetylcholine in 1:10,000–1:50,000 concentration. In every instance there was a definite second contraction in response to strychnine,

and in seven of the experiments this equalled (see sample graph B)² or exceeded (see sample graph C) the initial response to acetylcholine.

The eserine-strychnine-sphincter system. The contraction of the sphincter by eserine alone follows essentially the pattern of its response to acetylcholine alone. Eleven experiments have been made upon eight strips, with the eserine in concentrations ranging from 1:25,000 to 1:100,000. One strip failed to respond. Two others behaved atypically—one contracted slightly, the other markedly, to strychnine. The other eight strips all relaxed under the influence of strychnine (see sample graph E), two of them with a slight initial contraction and two others (one of which is shown in graph F) with a contraction following upon the relaxation.

As has just been mentioned, a contraction following strychnine is atypical for an eserized preparation (only two experiments out of eleven). There is thus a difference in the effect of strychnine here as compared with the acetylcholine experiments, in which thirteen out of thirteen showed contraction following strychnine. The reality of this difference may be emphasized for the reader by pointing out that the experiments illustrated in graphs D and E were made upon the same sphincter strip, respectively before and after washing.

DISCUSSION. Strychnine, in concentrations up to 1:40,000, does not alter the length of otherwise unstimulated dilator and sphincter strips. It has not here produced a paralysis through toxicity, however, as is shown by the fact that the same concentrations cause a partial relaxation of the adrenalin-contracted dilator and a further contraction of the acetylcholine-contracted sphincter. That the strychninized muscle is in good physiological condition, and responding to the drug as a stimulus rather than as a poison, is further indicated by the increased contraction one sometimes obtains in eserine-contracted sphincter preparations—occasionally even following an initial relaxation which might be thought to be evidence of intoxication.

Neither the effect of strychnine on the dilator nor on the sphincter can be explained in terms of adrenergic or cholinergic without supplementary hypotheses. Strychnine has not been shown to depress any adrenergic effector (except the dilator), or to stimulate any cholinergic effector (except the sphincter). But the drug has been found to depress some cholinergic mechanisms, namely in the superior cervical sympathetic ganglion (11) and at the myoneural junctions of striated muscle (12)—in both of which structures acetylcholine appears to be the actual trans-synaptic conductor.

The fact that strychnine usually relaxes an eserine-contracted sphincter preparation suggests that the prevalent explanation of the action of eserine may be inadequate. For, if eserine operates simply by antagonizing cholinesterase, one should expect the supposed residuum of acetylcholine to act synergistically with

² The minor secondary acetylcholine contractions shown in Graph B are quite characteristic, but such contractions never bring the muscle to a lesser length than that produced by the first, major contraction [see Sachs and Heath (10), p. 1385]. Hence one may be certain that the contraction following the administration of strychnine is indeed in response to that drug.

the strychnine, resulting in a contraction. For the same reason the direct parasympathetic-stimulating action of eserine, found by Manning *et al.* (13), although indistinguishable from that of acetylcholine, is probably different as to mode. Moreover, there is at least one situation—the ganglionic chain of the crayfish—in which strychnine has a depressant action in the utter absence of an acetylcholine mechanism (14). Such phenomena must be kept in mind in any attempt to fit the effects of strychnine into an inclusive explanation of the pharmacological responses of mammalian iris muscles.

CONCLUSIONS

1. Strychnine (1:40,000 or 1:50,000) produced neither contraction nor relaxation in 13 albino rabbit sphincter strips and 6 dilator preparations.

2. In 22 out of 23 experiments with adrenalin-contracted dilator strips, strychnine produced a relaxation, though never one sufficient to restore the original length of the muscle.

3. Cervical sympathetic ganglionectomy fails to abolish the effect described in 2.

4. In 13 experiments with acetylcholine-contracted sphincter strips, strychnine produced a second contraction which, in 7 experiments, equalled or exceeded the initial response to acetylcholine.

5. Among 11 experiments with eserine-contracted sphincter strips, strychnine had no effect upon one strip, yielded further contraction in 2, and relaxed 8 (with slight initial contraction in 2 and with subsequent contraction in two others). Relaxation is the prevailing response (contrast 4,) suggesting a considerable difference in the actual mode of action of the ordinarily like-acting eserine and acetylcholine.

6. The effects of strychnine on the isolated iris muscles cannot at present be related to their known adrenergic and cholinergic mechanisms, and the possibility exists that strychnine acts quite independently of those mechanisms.

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OBSERVATIONS ON THE CHRONIC TOXICITIES OF PROPYLENE GLYCOL, ETHYLENE GLYCOL, DIETHYLENE GLYCOL, ETHYLENE GLYCOL MONO-ETHYL-ETHER, AND DIETHYLENE GLYCOL MONO-ETHYL-ETHER

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In a previous publication from this laboratory (1) an extensive investigation of the acute toxicity of some glycols and derivatives was reported. Microscopic observations of tissue changes caused by acute doses were described, and a bibliography of the literature dealing with the pharmacological and toxicological properties of the glycols which had been reported up to that time was appended. At that time we stated that chronic toxicity studies of some of the glycols and their derivatives were in progress. The results of these studies are the subject of the present report. As is nearly always the case with investigations of this nature, it is obvious that further work is indicated on these same compounds and others of this series, but the results are of such a nature that it seems advisable to present them at this time for the information of others who are working in this field.

Propylene glycol has already been investigated to some extent from the subacute and chronic standpoint (2, 3, 4, 5, 6, 7, 8, 9, 10). In most instances the propylene glycol was administered in the drinking water in concentrations as high as 10 per cent. One group administered it daily by stomach tube, another group fed it as fatty acid esters mixed in the diet, and still another group fed propylene glycol as a substitute for part or all of the carbohydrate of the stock diet. In the chronic toxicity studies of diethylene glycol it has been given in drinking water (5, 7, 11, 12, 13), by stomach tube (14), and as fatty acid esters (3). The chronic effects of ethylene glycol have been observed after administration in drinking water (5, 7, 15), as fatty acid ester (3), and by inhalation of the vapors (16). In all the investigations of propylene glycol no noticeable effects of a deleterious nature have been noted except in those cases where large acute doses were administered (1). In all the cases where diethylene glycol has been studied the mortality has been high and pathological changes marked except when it was administered in very low concentrations in drinking water. In the case of ethylene glycol the results have been similar to those obtained from diethylene glycol, except where the animals were exposed to the vapors, in which case no toxic effects for rats and mice were noted for the concentrations studied. The only other investigations of the chronic toxicity of glycols that have been reported are those of trimethylene glycol (17), and of triethylene glycol (18).

EXPERIMENTAL PROCEDURE. Whereas the acute toxicity studies previously reported (1) were made on several species of animals, the chronic studies herein reported are limited to a single species, namely the albino rat from our own colony of an inbred strain. The breeding colony and the experimental animals were all housed in quarters which are kept at constant temperature and humidity. The animals for these experiments were selected in groups of 4 litter mates of the same sex and placed on experiment at 3 weeks of age. Three of the 4 litter mates were each placed on diets containing one of the substances under investigation. The fourth, serving as a control, received the basic diet only. Twenty litters were used in any one experiment which means that 20 animals were used on each experimental substance and that each animal had 3 litter mates, 2 of which were each on a different experimental substance, and 1 on the basic diet. By this means the effects of each of 3 substances could be compared on litter mate animals as well as with the litter mate control. The 3 glycols were compared in one series of 20 litters whereas the mono-ethyl-ethers of ethylene glycol and diethylene glycol were studied as a part of another series. The third substance of this latter series was not of a similar nature to the glycols and is not being reported at this time. The 20 animals on each of the 3 glycols were divided into 2 groups of 10 each, 1 group being used at each of the 2 levels at which these substances were studied. There were 6 males and 4 females in any one group of 10 animals. The substances being investigated were incorporated in the basic diet which has been described elsewhere (10). Ethylene glycol was fed at levels of 1 per cent and 2 per cent and the diethylene glycol was fed at equimolecular concentrations, or 1.71 per cent and 3.42 per cent. The propylene glycol was fed at levels of 2.45 per cent and 4.9 per cent, the lower concentration being the molecular equivalent of the 2 per cent ethylene glycol and the higher percentage twice this quantity. The ethylene glycol mono-ethyl-ether and the diethylene glycol mono-ethyl-ether were fed at levels of 1.45 and 2.16 per cent respectively, which are equivalent in molecular concentration to the 1 per cent ethylene glycol. These two substances were fed only at the one level. Litter mate male animals were placed on each of these and on the basic diet in groups of 20 animals on each diet. All animals were placed in individual cages and given free access to their respective diets and water. The weights and food consumption were determined at weekly intervals.

RESULTS. *Growth rate and food consumption.* When the weekly growth rates and weekly food consumptions were plotted for all of the experimental and control animals and the data analyzed for the first 55 weeks of the experiment the differences between the control and experimental animals were not statistically significant.

Survival. Statistical tests cannot be applied to the mean survival time since all animals were sacrificed at the conclusion of 24 months and it is not known how much longer they might have lived.

The number of animals surviving 2 years does not give a true picture because in the case of the rats receiving 2 per cent ethylene glycol the deaths are distributed over both years while in the control groups there were very few deaths until well into the second year. Accordingly the number of rats surviving the first year of the experiment was taken as a criterion of relative toxicity. Only in the case of the 2 per cent level of ethylene glycol is there a significant difference from the mortality of the control group. Seven of the 10 experimental animals died whereas all of the litter mate controls survived the first year. The chances are less than 2 in 1000 ($P = .0016$) that the consumption of 2 per cent ethylene glycol has not influenced the mortality of rats in the first year of the experiment. Gross and microscopic examinations of

the tissues of those animals which failed to survive provided no obvious explanation for this greater mortality.

Pathological changes. Inasmuch as a pathologist was not available until near the end of the experiment only 50 of the 100 experimental rats were microscopically examined. Therefore the 50 experimental animals studied are to a certain degree selected, since they are essentially those surviving the longest. There was also some selection of the ethylene glycol animals because those with stones in the bladder were always examined and the others sometimes discarded. Only 10 of the 40 litter mate controls were examined because of limited facilities and because of the examinations of large numbers of other control animals which had been fed the same basic diet. Microscopic sections of the lung, heart, liver, spleen, kidney, adrenal, and testis were made routinely; pancreas, stomach, intestines and lymph nodes were sectioned in about half of the animals, and other organs were occasionally sectioned.

Two outstanding lesions were observed. These were the production of laminated mulberry bladder stones from 0.8 to 1.7 cm. in diameter in 8 male rats given ethylene glycol and diethylene glycol (fig. 1), and testicular enlargement, edema and tubular atrophy in fully two-thirds of the animals that had received ethylene glycol mono-ethyl-ether (fig. 2). The latter lesion has not to our knowledge been reported in rats under any condition and bladder stones such as those seen in our rats have not been mentioned in previous reports on the chronic toxicity of the glycols. Table 1 briefly summarizes the pathological lesions found in the various groups of rats that were examined microscopically.

The testicular lesions were more often bilateral than unilateral. They consisted of marked interstitial edema and marked tubular atrophy, with the weight of one testis without its epididymis increased to as much as 7 grams instead of the normal 2 grams. The group of rats on ethylene glycol mono-ethyl-ether was outstanding in this respect, and the group on diethylene glycol mono-ethyl-ether was intermediate between this and the other groups. We have not yet been able to determine the pathogenesis of the lesions.

Large urinary calculi were grossly observed in a total of 6 animals fed ethylene glycol; 4 of these received one per cent ethylene glycol, the others 2 per cent. The difference in the number of stones observed in two groups of animals may probably be explained by the longer survival time of those animals receiving one per cent ethylene glycol with the diet. Large calculi were likewise observed grossly in 3 animals given diethylene glycol, each bladder containing 2 stones of approximately the same size; in the animals that received ethylene glycol several small stones frequently accompanied a single large one. Marked chronic cystitis accompanied the bladder stones to a greater degree in the ethylene glycol group than in the diethylene glycol group. Small colorless crystalline concretions, often very numerous, were found in the kidney tubules of 7 of our animals; the microscopic appearance and in one case crystallographic examination showed that calcium oxalate was the chief component. These concretions occurred in rats both with and

without bladder stones. Somewhat similar concretions have been found previously (20, 21).

Other chronic renal lesions consisted of tubular atrophy with less glomerular atrophy; tubular casts, usually hyaline, and slight degrees of lymphocytic infiltration and fibrosis. In the group of animals receiving ethylene glycol, because of the presence of bladder stones and renal oxalate concretions, the renal lesions were more severe than in the other groups. The chronic hepatic

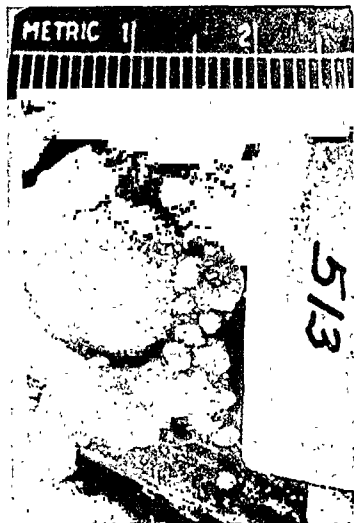


FIG. 1. ONE LARGE AND SEVERAL SMALLER BLADDER STONES REPRESENTATIVE OF THOSE FOUND IN SEVERAL OF THE ANIMALS RECEIVING ETHYLENE GLYCOL AND DIETHYLENE GLYCOL. THE LARGE STONE ALMOST FILLS THE OPENED AND THICKENED BLADDER.

lesions consisted of diffuse or centrilobular atrophy, bile duct proliferation and fatty degeneration. In each of these organs more than one of the conditions mentioned were often present. The lungs, heart, spleen, lymph nodes, pancreas, stomach, intestines, and adrenals of the rats on the various diets containing added experimental substances showed no lesions beyond those in the control animals.

Chemical analysis of the urinary concretions. One of the 2 stones found in

the bladder of an animal that received diethylene glycol, and stones or portions of stones from 3 of the animals receiving ethylene glycol, were chemically analyzed. The principal constituents are shown in table 2. When the stone from the animal receiving diethylene glycol was rinsed with water it was found to be soft and it began to crumble. On further agitation with water about 40 per cent of the entire stone was readily separated from the inner portion, which proved to be very hard and compact. The 2 portions of this



FIG. 2. TESTES OF RATS SHOWING LESIONS REPRESENTATIVE OF THOSE COMMONLY OBSERVED IN ANIMALS RECEIVING ETHYLENE GLYCOL MONO-ETHYL-ETHER, AND OCCASIONALLY IN OTHER ANIMALS. THE STRUCTURE BETWEEN THE LARGE EDEMATOUS TESTES IS THE DISTENDED URINARY BLADDER

stone were separately analyzed. All stones were analyzed for calcium, oxalate, phosphate, and carbonate ions. In those cases in which equivalent quantities of oxalate and calcium were found in the same stone the values have been reported as calcium oxalate. This was true for all of the stones produced by the ingestion of ethylene glycol and also for the inner portion of the stone from the animal receiving diethylene glycol. Tests were made for the presence of other constituents in these stones but since they were found only in traces they are not included in the table. Measurable quantities of phos-

phates were found only in the outer portion of the stone from the animal receiving diethylene glycol and in this material the oxalate and calcium contents were very low. As may be seen from table 2, not all of the constituents of the stones have been accounted for and we were unable to identify the remaining percentage. The stones were dried at a temperature of 200 to 250°C. for several hours before being analyzed and hence should have lost the water of hydration which usually is present in calcium oxalate. The relatively

TABLE 1

SUBSTANCE	BLADDER STONES	OXALATE CONCRETIONS IN KIDNEYS	ENLARGED EDEMATOUS TESTES*	CHRONIC KIDNEY DAMAGE*	CHRONIC LIVER DAMAGE*
Ethylene glycol	6†	6	None	Marked	Slight
Diethylene glycol ..	3		None	Slight	Slight
Propylene glycol			None	None	Slight
Ethylene glycol mono-ethyl-ether			Two-thirds of animals	Slight	None
Diethylene glycol mono-ethyl-ether		1	Few	None	Slight

* Beyond the number or degree in control animals

† One of these was a 5 mm. ureteral stone, in an animal receiving 2 per cent ethylene glycol in the diet.

TABLE 2

Composition of stones found in the bladders of rats which had ingested ethylene glycol or diethylene glycol

SUBSTANCE	DRY WEIGHT	CALCIUM OXALATE
	mgm	per cent
Ethylene glycol	35.2	82.5
Ethylene glycol .	10.3	81.2
Ethylene glycol	24.0	80.4
Diethylene glycol* (inner portion)	52.5	92.9

* The outer portion of this stone weighed 30.9 mgm. and contained 11 per cent of calcium, 7.3 per cent of oxalate ion, and 75.4 per cent of phosphate ion.

constant value of approximately 80 per cent of calcium oxalate in the stones indicates a rather uniform character of formation.

DISCUSSION AND SUMMARY

The results of this investigation illustrate clearly the advantage of the long-time chronic toxicity study. The results obtained by continuing the experiment for two years that would not have been noted if it had been discontinued at the end of one year were the occurrence of urinary calculi in each of the series of the animals receiving ethylene glycol and diethylene glycol. It is possible also that the microscopic lesions and testicular enlargement observed would not have been as distinctly different between the experimental

and control animals if the experiment had been continued for only one year. As we have emphasized elsewhere (22) chronic toxicity studies should be continued over long periods of time especially in the case of those substances that are to be included in the diet of man or are to be administered as therapeutic agents or applied as cosmetics repeatedly over long periods of time.

Although the actual number of urinary calculi found in this series of animals is small the significance of their occurrence is increased by the fact that over a thousand additional animals from our colony have been fed the same basic diet for control and experimental studies and in no case have such stones been found. The higher incidence of stones at the lower level of intake of ethylene glycol has already been referred to. Stones were found only in male animals, 6 in the ethylene glycol series and 3 in the diethylene glycol series. Although the number of male animals used predominates, the chances are less than 5 in 1000 ($P = 0.0048$) that the sex of the animal does not influence the occurrence of stones. The presence of urinary calculi composed primarily of calcium oxalate following the ingestion of diethylene glycol is almost conclusive proof that in the rat the ether linkage of diethylene glycol is broken and the end products so formed are converted to oxalic acid. The only alternative explanation, which is not very plausible, is that in these particular animals stones were formed from the very small amount of oxalic acid present in the basic diet.

Fully two-thirds of the animals on ethylene glycol mono-ethyl-ether showed marked testicular enlargement, edema and tubular atrophy. This condition occurred less frequently in the group on diethylene glycol mono-ethyl-ether and only occasionally in the other experimental groups and in the control groups. Chronic kidney damage was marked and renal concretions of calcium oxalate frequent only in the case of those animals receiving ethylene glycol. The group of animals receiving propylene glycol differed only very slightly from the controls.

Some of the important questions left unanswered by this investigation are obvious, namely, do other species respond in similar manner, and what are the lowest levels that can be ingested without the occurrence of stones and without effects on the vital organs, such as the liver, kidney, and reproductive organs? Until these questions have been answered the safety of the repeated use in any quantity of those substances producing such effects has not been established.

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THE SPASMOLYTIC AND LOCAL ANESTHETIC ACTION OF SOME DERIVATIVES OF FLUORENE-CARBOXYLIC ACID AND RELATED COMPOUNDS¹

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The clinical usefulness of atropine as a spasmolytic agent is greatly impaired by its undesired side-effects. For this reason various synthetic compounds are being tried as substitutes for atropine, and one of these is the diphenylacetic acid ester of diethylaminoethanol (Trasentin). In a previous study of local anesthetics (1) it was shown that the introduction of a carbon-carbon linkage between two benzene rings results in a marked decrease in toxicity with no decrease or only a slight decrease in potency. This phenomenon led to the synthesis of the diphenylene-acetic (fluorene-9-carboxylic) acid ester of diethylaminoethanol, and other related compounds, prepared by R. R. Burtner and J. W. Cusic at G. D. Searle and Company. Spasmolytic and local anesthetic actions, and toxicity of these compounds have been studied.

EXPERIMENTAL. Spasmolytic action was determined on the isolated intestine, according to the technic of Magnus, in three ways. Neurotropic action was tested on the rabbit's intestine after spasm was produced by $10^{-6.2}$ acetylcholine bromide. The musculotropic action was determined on the isolated rabbit's intestine against spasm caused by 10^{-4} barium chloride, and on the isolated guinea pig's intestine against spasm caused by 2×10^{-6} histamine acid phosphate. The loops of rabbit's intestine were suspended in Ringer-Locke solution at 37°C., and those of the guinea pig's intestine in Tyrode solution at 35°C. The potency is expressed in table 1 as reciprocal activity ratio in relation to compound 23, which has the most favorable therapeutic coefficient. As seen in the typical records shown in figure 1, approximately equal relaxation after the three spasmogenic agents is produced by concentrations of 1.28×10^{-7} , 4×10^{-6} , and 9.3×10^{-7} of the spasmolytic agent.

Musculotropic action is usually tested against spasm produced by barium ions. In our experience this method is not very sensitive to concentration differences of spasmolytic agents, and it is often difficult to obtain equal responses on repeated administration of Ba. Equal responses to histamine can be reproduced with great ease, and concentration differences of 20 per cent of the spasmolytic agent can usually be detected, while against Ba spasm, concentrations differing by 50 per cent are seldom detectable. The activity ratios found with both methods agree fairly well with some exceptions. Atropine is much

¹ This investigation has been aided by a grant from G. D. Searle and Company, Chicago, Ill.

² All such figures express the concentration in grams per cc.

more potent against histamine than it is against Ba, while papaverine antagonizes the spasmogenic agents equally.

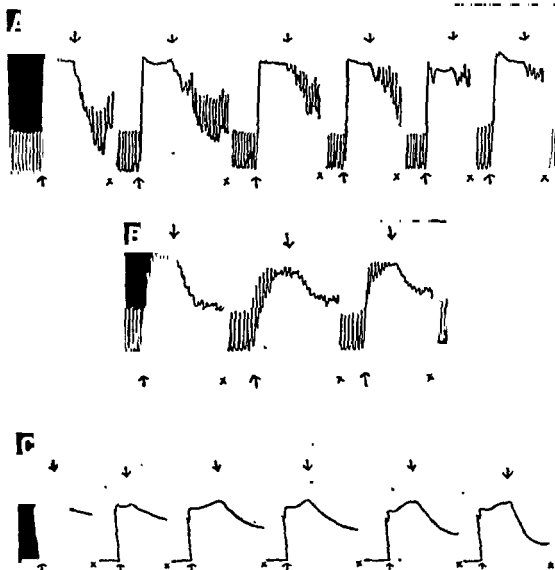


FIG. 1. A Rabbit ileum. At ↑ acetyl choline, 10^{-4} . At ↓, from left to right, diethylaminoethyl fluorene-9-carboxylate, 1.6×10^{-7} , 1.44×10^{-7} , 1.28×10^{-7} , 1.12×10^{-7} , 9.6×10^{-8} , 8×10^{-8} . At x, washout.

B. Rabbit ileum. At ↑ barium chloride, 10^{-4} . At ↓, from left to right, diethylaminoethyl fluorene-9-carboxylate, 8×10^{-8} , 6×10^{-8} , 4×10^{-8} . At x, washout.

C Guinea pig ileum. At ↑, histamine, 2×10^{-6} . At ↓, from left to right, diethylaminoethyl fluorene-9-carboxylate, 6.6×10^{-7} , 8×10^{-7} , 9.3×10^{-7} , 1.06×10^{-6} , 1.2×10^{-6} , 1.33×10^{-6} . At x, washout.

The local anesthetic action was determined on the rabbit's cornea by applying the solutions for one minute and testing the reflex at one minute intervals. The results recorded in table 1 show the duration and depth in three arbitrary degrees of intensity.

TABLE I

NO.	ACID	ALCOHOL	SPASMOLYTIC ACTIVITY RATIOS			ANESTHETIC ACTIVITY			TOXICITY: DOSE IN GM./KGM.											
			Acetylcholine	Histamine	Barium	Conc.	Duration per cent min.	Depth	LD ₅₀	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
1	<i>l</i> -Phenylacetic	<i>2</i> -Diethylaminoethanol	7	2	2	1	8	++	0.22		0/8	3/8	7/8							
2	<i>2</i> -Biphenylacetic	<i>2</i> -Diethylaminoethanol	20	1	1.5	1	14	+++	0.20		0/6	3/6	6/6							
3	Diphenylacetic	<i>2</i> -Diethylaminoethanol*	6	1.5	1.5	1	10	++	0.24		0/7	4/16	13/16	7/7						
4	Diphenylacetic	<i>2</i> -Dimethylaminoethanol	8	1.6	1.5	1	15	+++	0.26		0/6	5/6	4/4							
5	Diphenylacetic	<i>l</i> -Methyl-4-hydroxypiperidine	1.8	0.5	1				0.18	0/4	1/6	3/6	4/4							
6	Diphenylacetic	<i>l</i> , <i>2</i> , <i>6</i> -Trimethyl-4-hydroxypiperidine	4.6	2	2				0.3		0/6	3/6	4/4	4/4						
7	Diphenylacetic	<i>l</i> - <i>n</i> -Butyl-4-hydroxypiperidine	10	1.5	1	1	43	+++	0.15		0/6	5/6	6/6							
8	Diphenylacetic	<i>l</i> - <i>β</i> -Phenethyl-4-hydroxypiperidine	>10	3.7	4				0.46		0/6	1/6	4/6	6/6						
9	Diphenylacetic	<i>2</i> -Diethylaminoethanolamine	10	2.5	2.5	2	24	+++	0.15		0/6	5/6	6/6							
10	<i>2</i> , <i>2</i> -Diphenylacetic	<i>2</i> -Diethylaminoethanol	6	1.7	1	1	28	+++	0.14		0/6	6/6	4/4							
11	<i>3</i> , <i>3</i> -Diphenylacetic	<i>2</i> -Diethylaminoethanol	>10	10	10	1	0	0	0.71		0/6	1/6	6/6							
12	Diphenylmethylethylcarbamate	<i>2</i> -Diethylaminoethanol	6.5	1.5	1.5	2	41	+++	0.13		0/6	6/6	5/6							
13	Diphenylchloroacetic	<i>2</i> -Diethylaminoethanol	0.7	0.7	1.5	1	15	+	0.076	0/6	5/6	6/6	6/6							
14	Diphenylglycidyl (benzylidene)	<i>2</i> -Diethylaminoethanol	0.7	0.7	2	1	22	+++	0.076	0/6	5/6	6/6	6/6							
15	Di(4-methoxyphenyl)glycollic (<i>p</i> -anisic)	<i>2</i> -Diethylaminoethanol	10	6	5	1	12	++	0.14		0/6	6/6	6/6							
16	<i>2</i> , <i>2</i> -Diphenyl-4-hydroxypropionic	<i>2</i> -Diethylaminoethanol	6	5	5	1	18	++	0.14		0/6	6/6	6/6							
17	<i>l</i> , <i>l</i> -Diphenyl-2-hydroxypropionic	<i>2</i> -Diethylaminoethanol	5	2	2	1	18	++	0.26		0/6	4/6	6/6							
18	<i>l</i> -Phenyl-2-hydroxypropionic (d,l-tropic)	<i>2</i> -Diethylaminoethanol	1	20	20	1	0	0	0.46		0/6	2/12	9/12	10/10						
19	<i>l</i> -Phenyl-2-hydroxypropionic (d,l-tropic)	<i>3</i> -Diethylamino-2,2-dimethyl propanol†	15	30	20						0/6	1/6	6/6							
20	<i>l</i> -Phenyl-2-hydroxypropionic (d,l-tropic)	Tropine†	0.14	4	20				0.24		0/6	1/6	6/6							
21	Atropine	<i>2</i> -Diethylaminoethanol	30	3	4	1	0	0	0.25		0/6	6/6	6/6	6/6						

22	Atropine	1-Methyl-4-hydroxy-piperidine	30	2	4	1	0	0	0.13	0/6	1/6	6/6	4/16	11/12				
23	Fluorene-9-carboxylic	2-Diethylaminoethanol	1	1	1	1	15	+++	0.32		0/12	1/14	4/16	4/4				
24	Fluorene-9-carboxylic	3-Diethylaminopropanol	4	5.5	5	1		+++	0.22	0/4	1/5	4/4	4/4					
25	Fluorene-9-carboxylic	4-Diethylaminopropanol	>10	4.5	2	1	33	+++	0.33		0/5	2/6	4/5					
26	Fluorene-9-carboxylic	5-Diethylaminopropanol	>12	9	5			+++	0.40		0/6	3/6	6/6	4/4				
27	Fluorene-9-carboxylic	6-Monoisobutylaminoethanol	5	2.5	2	1	20	+++	0.20		0/6	3/6	6/6					
28	Fluorene-9-carboxylic	7-Methyl-4-hydroxy-piperidine	1	0.6	1				0.15	0/7	3/6	5/5						
29	Fluorene-9-carboxylic	1,2,6-Trimethyl-4-hydroxy-piperidine	2.8	1	1	1	26	+++	0.23	0/6	2/6	5/6	4/4					
30	Fluorene-9-carboxylic	1,2-Phenethyl-4-hydroxy-piperidine	6	3.5	4	1	19	++	0.23		0/6	4/6	6/6	4/4				
31	Fluorene-9-carboxylic	2-Diethylaminoethanol	12	5	6	2	40	++	0.076	0/6	5/6	6/6						
32	Fluorene-9-acetic	3-Diethylaminoethanol	11	1	1	1	19	++	0.26		0/6	6/6						
33	Fluorene-9-acetic	4-Diethylaminoethanol	>10	10	1	0	0	0	0.45		0/8	2/3	8/3	1/4	2/5	3/4		
34	Fluorene-9-hydroxy-9-carboxylic	5-Diethylaminoethanol	1.2	2	3	1	13	++	0.13	0/6	2/3	8/3						
35	Fluorene-9-amino-9-carboxylic	6-Diethylaminoethanol	20	9	10	1	0	0	0.13	0/6	1/6	6/6	3/5					
36	1-Naphthoic	7-Diethylaminoethanol	50	3	4	1	12	++	0.45				0/6	2/6	4/6	5/6		
37	1,4,5,8-Tetrahydro-1-naphthoic	8-Diethylaminoethanol	40	3	2	1	6	+	0.45				0/6	2/6	4/6			
38	Anthracene-10-carboxylic	9-Diethylaminoethanol	10	3	3	1	53	+++	0.29		0/6	0/6	4/6	6/6				
39	9,10-Dihydroanthracene-10-carboxylic	10-Diethylaminoethanol	5	0.03	1	1	33	+++	0.15		0/6	6/6						
40	Acridene-9-carboxylic	11-Diethylaminoethanol	30	4	3.5				0.29				4/6	6/6				
41	Di(1-naphthyl) acetic	12-Diethylaminoethanol	>50	20	>20				0.35				2/6	4/6	6/6			
42	Di(1-naphthyl) glycollic	13-Diethylaminoethanol	50	4	2				0.44				0/5	2/6	5/6	4/4		
43	Di(1-naphthyl) acetic	14-Diethylaminoethanol	200	40	3				0.63				0/6		1/6	3/6		
44	Di(1-naphthyl) glycollic	15-Diethylaminoethanol	>100	5	2				0.15		0/6	5/6	6/6					
45	Papaverine		20	1.5	1.5													6/6

* "Triphenyl."

† "Syntran."

‡ Atropine.

All compounds except 19, 20 as hydrochlorides, 19 as phosphate, 20 as sulfate.

Mortality ratios = dead animals/animals used.

Toxicity of all compounds was determined by intraperitoneal injection in white mice (table 1). The values of LD_{50} were found by interpolation. In addition the toxicity of compounds 3 and 23 was found by various routes of injection in mice and dogs (table 2).

Relaxing action was determined by many of the compounds on the small intestine *in situ*, in the rabbit, guinea pig, and dog. While precisely quantitative comparisons of all compounds were not obtained, the results are in agreement with the comparisons *in vitro* against acetyl choline. In the rabbit, urethane anesthesia, sometimes with the addition of ether, was used and contractions of the ileum recorded by the method of Trendelenburg (2). Sixteen experiments

TABLE 2

DOSE	ORALLY IN MICE		SUBCUTANEOUSLY IN MICE		INTRAVENOUSLY IN MICE		INTRAVENOUSLY IN DOGS	
	# 3	# 23	# 3	# 23	# 3	# 23	# 3	# 23
gm. per kgm.								
0.010						0/6		
0.0125						3/8		
0.015					0/4	6/6		1/6
0.020					3/8	4/4		3/6
0.025					4/6			4/6
0.030								6/6
0.040							0/6	
0.050							3/6	
0.060							6/6	
0.400				0/6				
0.500				1/10				
0.600		0/6	0/10	1/10				
0.700				3/10				
0.800	1/10	0/8	5/10	5/10				
0.900				10/10				
1.000	7/12	3/10	7/10					
1.200	4/6	5/10	3/4					
1.500	4/4	10/11						

Mortality ratio = dead animals/animals used.

were done, and the results are collected in table 3. A typical record is seen in figure 2.

In four guinea pigs anesthetized with urethane, the motility of the ileum was measured by Straub's method (3). This method measures the threshold of distending pressure at which motility appears. The results are seen in table 3, and a typical record is seen in figure 3.

In nine dogs, anesthetized with barbital, barbital and morphine, urethane, or chloralose, the motility of the small intestine was recorded by a balloon inserted within the lumen. The results are recorded in table 3, and a typical record is shown in figure 4, A. In some of the dogs and rabbits, the administration of physostigmine to augment intestinal contractions appeared not to influence the effects of the spasmolytic agents.

TABLE 3
Comparisons of intestinal relaxing effect

RABBIT	GUINEA PIG	DOG
$\#3 = \#4$ $2 \times \#3 = \#5 \quad \#5 > \#6$ $2 \times \#3 = \#16$ $\#3 < \#17$ $\#13 = \#14$ $\#14 > \#18$ $5 \times \#17 = \#18$ $\#18 > \#19$ $\#23 > \#3$ $\#23 = 10 \times \#24$ $\#23 > 10 \times \#25, \#26, \#27$ $\#23 = \#28 = \#5$ $\#28 > \#29$ $\#23 > 10 \times \#32$ $\#23 > 10 \times \#35$ $\#23 = 6 \times \#19$ $10 \times \#23 = \#20$	$\#5 > \#6$ $\#23 > \#3$ $\#23 = \#28$ $\#28 > \#5$ $\#23 > \#19$	$\#3 < \#5 > \#6$ $\#23 > 5 \times \#3$ $\#23 = \#5 \quad \#23 < \#28 > \#29$ $\#28 > \#5 \quad \#29 > \#6$ $\#23 > 10 \times \#19$ $\#23 < \#20$ $\#23 > 10 \times \#45$

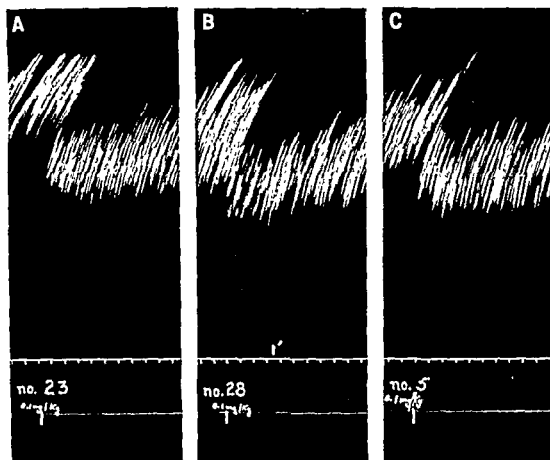


FIG. 2. RABBIT, URETHANE ANESTHESIA INJECTIONS INTO JUGULAR VEIN
From the top down, ileum, in situ, upstroke = contraction; time in minutes; signal of injection.

A. Diethylaminoethyl fluorene-9-carboxylate, 0.1 mgm per kgm.
per kgm.

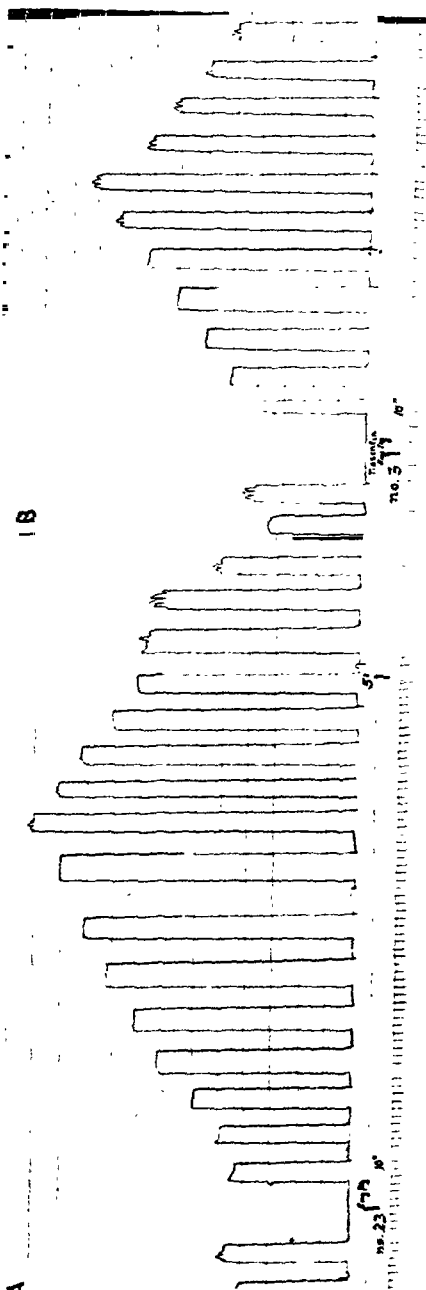


FIG. 3. GUINEA PIG, URETHANE ANESTHESIA. INJECTIONS INTO JUGULAR VEIN on the top down, pressure in loop of ileum in centimeters of water, signal of injection, time in ten seconds. Diethylaminoethyl fluorene-9-carboxylate, 5 mgm. per kgm. Diethylaminoethyl diphenylacetate, 5 mgm. per kgm. tween A and B, nine minutes.

Since compound 23, diethylaminoethyl fluorene-9-carboxylate hydrochloride, seemed to have the most favorable balance of spasmolytic activity and toxicity, its actions were studied further. It relaxes the smooth muscle of other organs, as shown in figure 4, B for the detrusor of the urinary bladder, and the uterus. It has no marked broncho-dilator action. This compound has no vasodepressor action in doses adequate to relax the small intestine (fig. 4, A). In antagonizing the vasodepressor action of acetyl-choline, it is one hundred and thirty times

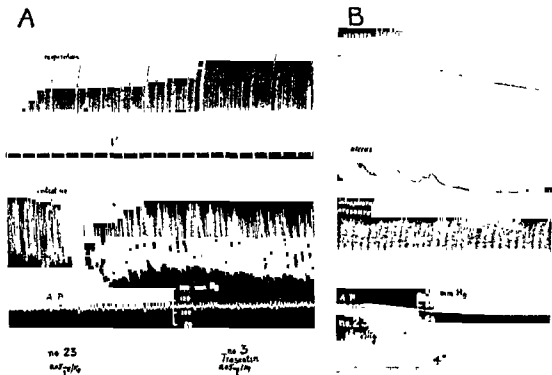


FIG. 4 A Dog, barbitol and morphine anesthesia. Injections into femoral vein.

0.05 mgm. per kgm.

B Spinal dog, artificial respiration. Injection into femoral vein. From the top down, pressure in urinary bladder, uterus, upstroke = contraction; intrapleural pressure; carotid arterial pressure; time in four second intervals. At signal, diethylaminoethyl fluorene-9-carboxylate, 2 mgm. per kgm. Pilocarpine was injected previously.

weaker than atropine, as determined in three dogs by the method of Kuhl (4). "Trasentin" was found to be nine hundred times weaker than atropine.

The mydriatic action of this substance is five thousand times weaker than that of atropine, as shown in table 4. Its effect in preventing the sialogogue action of pilocarpine is more than one hundred times weaker than that of atropine, although stronger than that of "Trasentin" (fig. 5).

DISCUSSION. The systematic search for synthetically prepared spasmolytic agents has shown that spasmolytic action does not depend on the presence in the molecule of either tropic acid or tropine. The esterification of other organic acids with a basic alcohol results in spasmolytic activity. As concerns the acid,

Halpern (5) showed that the esters of both aromatic and aliphatic acids possess spasmolytic activity if the latter acid has at least six carbon atoms. Of the large series of compounds studied by Halpern, diethylaminoethyl esters of α -phenyl aliphatic acids were highly active, and in this series the peak of activity occurred with α -phenyl-valeric ester. We found this compound (no. 1) to be about as potent as the diphenylacetic ester, thus the introduction of a second phenyl group appears to confer no special properties. Propyl and phenyl

TABLE 4
Pupil size in millimeters

HOURS	LEFT EYE	RIGHT EYE	LEFT EYE	RIGHT EYE	RIGHT EYE	LEFT EYE	RIGHT EYE	LEFT EYE
0.0	Atropine sulf., 0.001% in left eye		#23, 5% in left eye		#23, 1% in right eye		#3, 5% in right eye	
0.5			5	2	3	4	9	4
1.0	10	7	5	1	5	5	10	3
1.5	10	5	5	2	3	3	10	3
2.0	11	5	5	2	2	2	6	3
2.5	8	4					4	3

The results of four separate experiments on the same cat on different days.

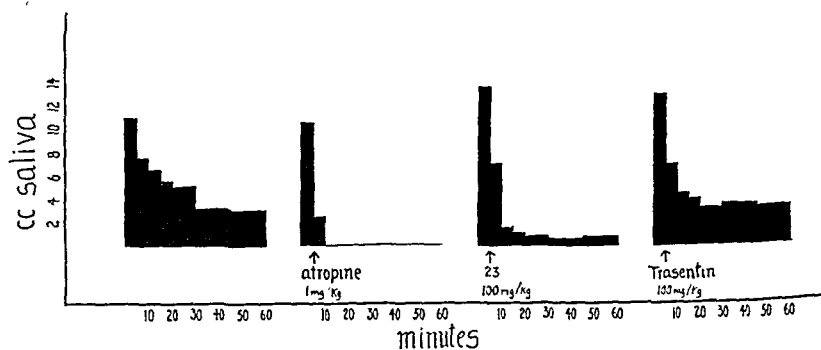


FIG. 5. SECRETION OF SALIVA

Four rabbits, urethane 1 gm. per kgm. per os; pilocarpine 15 mgm. per kgm. subcutaneously in each.

Ordinate: saliva in cc.

Abscissa: minutes.

From left to right: control, at arrows: subcutaneous injections of atropine 1 mgm. per kgm., diethylaminoethyl fluorene-9-carboxylate 100 mgm. per kgm. and diethylaminoethyl diphenylacetate 100 mgm. per kgm.

groups have been found to be approximately equivalent elsewhere, in the alkylammonium compounds studied by Raventos (6).

It has appeared with four pairs of compounds (3-23, 5-28, 6-29, 8-30) that the introduction of a carbon-carbon bridge between two benzene rings of the acid results in a marked increase in potency without significant increase in toxicity. In two instances (9-31, 14-34), this was not the case. Ring closure of the form seen with compounds 2-23 increased potency and decreased toxicity,

while that of the type found in 1—37 reduced both potency and toxicity. Spasmolytic potency is reduced by increasing the distance between the carboxyl group and the aromatic nucleus, by substitution on the aromatic nucleus, or by increasing its size to anthracene or acridine, or to dinaphthyl acetic acids instead of diphenyl acetic acid. The change from tropic to atropic acid results in a decrease in neurotropic potency, but an increase in musculotropic potency and toxicity, as is the case with atropine-apoatropine (7). Spasmolytic potency is reduced by increasing the size of the aminoalcohol beyond that of diethylaminoethanol. In general, the neurotropic potency seems to vary with changes in structure more widely than does musculotropic potency. Changes in local anesthetic activity appear to relate more closely to changes in musculotropic potency, than other properties.

SUMMARY

1. Forty-one compounds, esters of diphenylacetic and diphenyleneacetic acids, and related compounds, have been studied for spasmolytic and local anesthetic actions, and toxicity, in comparison with atropine, "Syntropan," "Trasentin," and papaverine.

2. Relaxation of spasm of the small intestine *in vitro* produced by acetylcholine ("neurotropic action") and relaxation of the spasm produced by histamine and barium ("musculotropic action") have been determined. The relaxing action on the small intestine *in vivo* in rabbits, guinea pigs, and dogs, has been observed. Changes in structure give a wider variation in neurotropic potency than in musculotropic potency.

3. Local anesthetic action is related more closely to musculotropic action than to other properties.

4. The introduction of a carbon-carbon linkage in esters of diphenylacetic acid, giving diphenyleneacetic acid (fluorene-carboxylic acid) derivatives, results, in most cases, in an increase in spasmolytic potency with little or no increase in toxicity.

5. The most promising compound, diethylaminoethyl-fluorene-9-carboxylate, has a spasmolytic potency varying from one-seventh to 20 times that of atropine, depending upon the test object. In antagonizing the vascular action of acetylcholine, atropine is 130 times stronger, in mydriatic action atropine is 5000 times stronger, and in antisialogogue action atropine is more than 100 times stronger than this compound.

6. Diethylaminoethyl fluorene-9-carboxylate warrants clinical trial as a spasmolytic agent.

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INFLUENCE OF DIET ON SULFANILAMIDE TOXICITY

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The administration of sulfanilamide and its related compounds occasionally results in acute toxic reactions with evidence of damage to various organs. These reactions have been well defined clinically but there have been few experimental studies on the factors responsible for drug intolerance. Information is lacking particularly with regard to subacute or chronic poisoning following prolonged administration. Smith, Lillie, and Stohlman (1) have recently reported that a low protein diet increases the susceptibility of rats to sulfanilamide as shown by a greater mortality rate and a higher incidence of anemia. There have been many reports of the influence of protein and carbohydrate dietaries on the action of various liver toxins (2). This becomes an important consideration in the management of war wounds because chemotherapeutic agents must be given over a prolonged period to people whose diet has been restricted by the rationing of foodstuffs. It seemed of interest, therefore, to study the metabolism of albino rats on high protein, high carbohydrate, and high fat diets while such animals were ingesting rather large amounts of sulfanilamide.

MATERIALS AND METHODS. Healthy young adult male albino rats weighing 130 to 180 grams and obtained commercially were used in all experiments. The animals were placed in metabolism cages which permit accurate measurement of daily water and food intake and urine volume output. The rats were divided up into four equal groups, each of which received a different diet. The composition of the diets is shown in table 1.

All the animals thrived and gained weight consistently on each of the above rations. After four to eight days on their respective diets all the rats were started on daily intragastric administrations of a suspension of 10 per cent sulfanilamide in 5 per cent gum acacia solution. This was administered through a soft no. 8 urethral catheter inserted into the stomach under very light and brief ether anesthesia. Attempts to pass stomach tubes without anesthesia in these untrained rats resulted in struggling with considerable trauma to the mouth and pharynx. Since such daily trauma interfered with the animal's eating, it seemed preferable to use a very small amount of ether. It should be pointed out that these experiments were performed without controls of the toxic effect of acacia alone.

In the first experiment a daily dosage of 1 gram sulfanilamide per kilogram of rat was administered, but in the subsequent two experiments the dose was increased to 1.5 grams per kilogram. On dosages of 2 grams per kilogram all the animals failed to take food and lost weight rapidly.

The water and food intake, urine volume, and weight were determined daily. Output of free and total sulfanilamide in the urine was determined every three to four days on the total urine volume of the period by the method of Bratton and Marshall (3). At intervals during the experiments all urines were examined for albumin by the nitric acid and heat and acetic acid tests.

After the drug had been given daily for three to four weeks all surviving rats were sacrificed twenty-four hours after the final dose of sulfanilamide. Under ether anesthesia, and while the heart was still beating, blood was withdrawn for prothrombin, hematocrit, and

sulfanilamide determinations. Prothrombin determinations were made by the method of Quick (4). Autopsies were done to discover any gross variation from the normal.

RESULTS. Twenty-four rats were used in each of three experiments. Forty of the 72 rats survived and were killed at the end of their respective experiments. Thirty-two of the rats either died or became so ill that they had to be killed prematurely. The mortality rate by diets is shown in table 2.

Most of the fatalities occurred fairly early in the experiment, after progressive weight loss, weakness, and emaciation. This was most marked in the group on the high carbohydrate diet (*no. 2*). Practically all of these rats lost weight,

TABLE 1

DIET	No. 1 CONTROL	No. 2 HIGH CHO	No. 3 HIGH PROTEIN	No. 4 HIGH FAT
	gm	gm	gm	gm.
Salt mixture*	4 0	4.0	4.0	4.0
Brewer's yeast	5 0	5 0	5.0	4.0
Casein	10 0	3.0	38 0	10.0
Crisco	22 8	12 8	12.8	40 8
Corn starch	53 0	75 0	40 0	40.0
Ol. percomorph	0 2	0.2	0.2	0.2

* The salt mixture used had the following composition:

CaCO ₃	31.5
MgCO ₃	7.6
KCl	23 0
NaH ₂ PO ₄ ·H ₂ O	25 8
Na ₂ CO ₃	5.1
Iron ammon. citrat.	2.0

TABLE 2

	DIED	SURVIVED	SURVIVED
			per cent
Diet no. 1—control	7	11	61.1
Diet no. 2—high CHO	11	7	38.8
Diet no. 3—high protein	5	13	72.2
Diet no. 4—high fat	9	9	50 0

became weak, and looked ill. They remained in poor clinical condition throughout the experiment with definite evidence of intolerance to the drug. Many died and the animals on this diet definitely did not tolerate the drug as well as the others.

Weight curves. The average weight change of the 40 rats which survived the experiments is shown in table 3, expressed as per cent gain or loss at the end of the experiment as compared with the weights of the animals the day administration of sulfanilamide was begun.

Liver. None of the animals developed clinical jaundice nor was bilirubin found

in the urine. Blood prothrombin determinations showed no significant differences between the different groups. All values were between 14 and 18 seconds in these experiments. At postmortem the rats fed the high fat diet (*no. 4*) uniformly showed fatty infiltration of the liver. Some of the rats on the high carbohydrate diet (*no. 2*) also showed varying degrees of fatty infiltration. Otherwise the livers showed no gross abnormalities. There was no evidence of cirrhosis.

Kidney. Nine of the original 72 animals developed gross hematuria at some period during the experiment. It appears significant that 5 of these were on the high carbohydrate diet (*no. 2*) and 4 on the high fat diet (*no. 4*). This occurred most often in the early days of the experiment. In 6 of the animals the hematuria was transient, lasting only a day or two and then spontaneously dis-

TABLE 3

	AVERAGE WEIGHT CHANGE PER ANIMAL
	<i>per cent</i>
Diet <i>no. 1</i> —control.....	+20.1
Diet <i>no. 2</i> —high CHO.....	-3.8
Diet <i>no. 3</i> —high protein.....	+34.4
Diet <i>no. 4</i> —high fat.....	+7.2

TABLE 4

	URINE PER 100 GRAMS OF RAT PER DAY	
	Control period before sulfanilamide	Average of all experiments with sulfanilamide
	<i>cc.</i>	<i>cc.</i>
Diet <i>no. 1</i> —control.....	1.8	6.6
Diet <i>no. 2</i> —high CHO.....	3.1	6.3
Diet <i>no. 3</i> —high protein.....	5.7	9.5
Diet <i>no. 4</i> —high fat.....	1.9	4.2

appearing. In 3 animals the hematuria was followed by death of the animals within twenty-four hours.

Only 2 of the rats developed albuminuria and this was slight and transient. One rat was on the control diet (*no. 1*), the other on the high carbohydrate diet (*no. 2*). At postmortem several of the rats showed kidneys which appeared to be somewhat enlarged but there were no other gross abnormalities.

Urine volume. In all experiments the animals on the high protein diet (*no. 3*) excreted a much greater volume of urine than any of the others and this was also true of the control period while the rats were on the special diets and before sulfanilamide was begun. This is shown in table 4. In these experiments the high protein rats excreted about 50 per cent more urine than those on the control and high carbohydrate diets (*no. 1* and *no. 2*), and more than twice as much as the animals on the high fat diet (*no. 4*).

Skin. An observation of considerable interest was that about one week after starting sulfanilamide therapy some of the rats began to lose their hair. This continued to the end of the experiment, at which time some of the rats were practically denuded. Almost all of the rats on diets *no. 1*, *no. 2*, and *no. 4* showed considerable loss of hair, most marked in the high carbohydrate group (80 per cent), followed by the high fat group (70 per cent), and the control group (60 per cent). Most of the animals on the high protein diet showed no hair loss, but a few exhibited this to a slight extent (20 per cent).

Gastro-intestinal tract. Some of the rats on the high carbohydrate diet developed transient diarrhea but the other groups showed well formed stools throughout. There was no other evidence of gastro-intestinal irritation except for occasional anorexia in some of the rats. Usually this was accompanied by an increased water intake. It was impossible to evaluate the rôle of acacia in producing these changes.

Many of the animals had large spleens which were very dark in color and firm in consistency. The adrenals showed no gross abnormalities in any of the rats.

TABLE 5

	AVERAGE BLOOD LEVELS 24 HOURS AFTER FINAL DOSE	
	Total sulfanilamide	Free (non-acetylated sulfanilamide)
	mg. per cent	mg. per cent
Diet <i>no. 1</i> —control.	11.3	8.2
Diet <i>no. 2</i> —high CHO	15.3	13.0
Diet <i>no. 3</i> —high protein	2.5	1.3
Diet <i>no. 4</i> —high fat	7.8	6.2

Blood sulfanilamide levels. The average blood levels for all surviving rats of total and non-acetylated sulfanilamide twenty-four hours after ingestion of the final dose is shown in table 5. The animals on the high protein diet showed uniformly lower levels than the others. About 41 per cent of the rats showed no acetylation of the drug in the blood stream as the level of the free form was exactly the same as the total. The other animals varied in the amount of acetylated drug from a trace to 43 per cent. There was no correlation with the diet of the rats.

Excretion of sulfanilamide in urine. Table 6 shows the average excretion of total and free sulfanilamide in the urine for all experiments, expressed as milligrams per 100 grams of rat per day. Marshall and Cutting (5) have shown that in the rat sulfanilamide is rapidly absorbed when given by mouth. The total amount of the drug excreted in the urine over the course of three to four weeks was not very different in the various groups of animals. It is believed that there was no essential difference in absorption of the drug from the intestinal tract on the different diets but that the differences in blood levels were due to differences in the rate of excretion of the drug. All of the rats showed some acetylation of

the drug in the urine, varying from 5 per cent to 60 per cent, but averaging from 30 per cent to 40 per cent.

Hematocrit. The average hematocrit of all surviving rats is shown in table 7. The animals on the high protein diet showed no anemia whatsoever, whereas those on the high carbohydrate diet developed a moderate anemia.

Discussion. It is obvious that the intake of a relatively large amount of casein appears to protect albino rats from the toxic actions of large amounts of sulfanilamide suspended in acacia. It permits the animals to gain weight and to continue growth and activity. Taking into consideration the weight curves of the animals that died prematurely, especially in the carbohydrate group, the differences are even more striking. The toxic effects upon the liver, kidney,

TABLE 6

	AVERAGE URINE EXCRETION IN MGS./100 GRAMS OF EAT PER DAY	
	Total sulfanilamide	Free (non-acetylated sulfanilamide)
	mg.	mg.
Diet no. 1—control.....	75.1	59.2
Diet no. 2—high CHO.....	73.2	63.4
Diet no. 3—high protein.....	86.1	68.5
Diet no. 4—high fat.....	63.4	41.3

TABLE 7

	AVERAGE HEMATOCRIT AT DEATH
	per cent
Diet no. 1—control.....	49
Diet no. 2—high HCO.....	41
Diet no. 3—high protein.....	54
Diet no. 4—high fat.....	47

skin, and hematopoietic systems are minimal in the animals ingesting large amounts of casein.

The mechanism by which this protective effect is exerted is of prime importance. It is a well-known fact that animals and humans on a high protein diet excrete a relatively large urine volume. This was found to be the case in these experiments, both before and after sulfanilamide was begun. The blood level of sulfanilamide was consistently lowest in all animals on the high protein diet. Sulfanilamide acts physiologically like any other foreign non-threshold crystalloid destined for proportionate excretion through the kidney. The general level of excretion through the kidney is increased in the animals on the high protein diet and the drug appears to be washed out of the blood stream non-specifically along with the excess nitrogenous waste products.

effects of a high protein diet. However, the excess protein unquestionably helps by maintaining optimal nutrition, conducive to rapid tissue repair. In this connection we were somewhat surprised by the poor showing made by the animals on the high carbohydrate diet, since the protein ration in these diets is sufficient for maintenance and growth of the animal. Actually, however, the carbohydrate rats were exposed to continuously higher blood levels of the drug, as noted above, and comparison is difficult.

There are many reports in the literature concerning the protective action of a high protein diet against hepato-toxins. It is clear that changes in renal function may be of considerable importance and must be considered in the interpretation of these observed phenomena.

There was very little difficulty from intubation of the animals. The technic is simple and effective. We do not believe that the amount of ether anesthesia is sufficient to affect materially the results of the experiments.

CONCLUSIONS

1. A high protein (casein) diet protects rats being given large amounts of sulfanilamide suspended in acacia daily, as shown by better survival, weight gain, condition of coat, physical activity, and clinical appearance. Drug intolerance is increasingly apparent on the general maintenance, the high fat, and the high carbohydrate diets.

2. An increased urine volume is associated with a greater elimination of sulfanilamide and a lower concentration of the drug in the blood of the rats on a high protein diet.

3. It is suggested that the greater tolerance afforded by the high protein diet is due to some extent to a more rapid excretion of sulfanilamide

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THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTIVITY OF FORTY-THREE NEW SYNTHETIC LOCAL ANESTHETICS¹

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The 43 compounds to be reported were synthesized in the Department of Organic Chemistry, Yale University, under the supervision of A. J. Hill, in collaboration with K. G. Pleger, R. B. Holmes, V. S. Salvin and G. L. Boomer. The chemical data will be published elsewhere. The compounds can be conveniently divided into four chemical groups: (A) alkamine esters of diphenylacetic acid, diphenylpropionic acid, and closely allied derivatives; (B) alkamine esters of phenyl alkyl or phenyl alkylene substituted acetic acid; (C) symmetrical alkamine esters of diphenylsuccinic acid; (D) derivatives of alkamine benzyl ethers.

METHODS AND INDICES. The compounds were tested for their efficacy as surface and block anesthetics. For testing surface anesthesia, the rabbit cornea was employed. Solutions were made of the hydrochloride salts in isotonic sodium chloride. The anesthetics were allowed to remain in contact with the cornea for two minutes after filling the conjunctival sac with the test solution. Following this interval, the anesthetic was removed by drainage and washing the eye with sodium chloride solution. The anesthetic thresholds and the duration of action of two per cent solutions of the various compounds were determined. Compounds failing to produce corneal anesthesia in two per cent solution were termed "inactive."

The activity of a number of the compounds as blocking agents was tested by the frog urostyle method (1, 2). Minimal anesthetic dose (mgm per gram for sensory anesthesia), the duration of anesthesia produced by this dose, and the dose required to maintain spinal anesthesia for 180 minutes were determined.

The irritating property of the individual compounds was estimated by careful observation of the rabbit eye. The ratings assigned in the tables are a composite index of hyperemia, edema, and corneal insult and are graded from one to four plus, the latter being indicative of intense vascular congestion, edema of the lids, and clouding and pitting of the cornea.

Minimal lethal dose (M.L.D.₁₀₀) was determined by intraperitoneal injection in mice. The large number of compounds and the small amount available of each precluded the use of large series of animals to ascertain toxicity, so that the figures presented in the tables can only be considered as close approximations. The intraperitoneal route of administration was chosen in order that the measurement of toxicity might reflect both the absolute toxicity of the compounds and their relative rates of destruction. Many members of the series are rapidly hydrolyzed so that subcutaneous injection would give a misleadingly

¹ Data appearing in this communication formed the basis of theses submitted by John M. Thomas, George A. Hahn and John M. Prutting in partial fulfillment of the requirements for the Degree of Doctor of Medicine, Yale University School of Medicine.

Expenses for this research were defrayed in large measure by a grant from the Calco Chemical Company.

low toxicity value, due to the close balance between rate of absorption and rate of destruction. On the other hand, intravenous toxicity determinations would err on the opposite side in that the desirable property of rapid rate of destruction is not reflected in the intravenous M.L.D.

Various indices were employed to gauge the relative anesthetic potency, toxicity and therapeutic efficacy of the different compounds. Cocaine was used as the basis of comparison. An index of anesthetic potency greater than unity represents activity greater than that of cocaine. An index of toxicity less than unity represents toxicity less than that of cocaine. Therapeutic ratios represent the indices of anesthetic potency divided by the index of toxicity. The therapeutic ratios for both surface and block anesthesia were calculated on the basis of mouse toxicity. Bieter, Harvey and Burgess (1932), in evaluating the therapeutic ratios of anesthetics as blocking agents after intrathecal injection in the frog, used the toxicity data for the frog obtained by this method of injection. However, it seemed more valid to use the mouse toxicity figures for the following reasons. Whereas the mechanism of nerve block is comparable in amphibia and mammals, the basis of toxicity is probably quite different. Also, after urostyle injection in the frog, the anesthetic produces death by contact depression of the central nervous system, whereas after parenteral injection in mice, the mechanism of toxicity is similar to that noted clinically after regional or spinal block, namely, absorption and general systemic toxicity.

RESULTS AND DISCUSSION. GROUP A. The compounds of this group are tertiary amino esters of diphenylacetic acid, diphenylpropionic acid and closely allied derivatives. The quantitative pharmacological data for the compounds are presented in table 1. The significant characteristics and relationships will be discussed for the individual compounds.


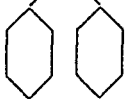
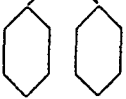
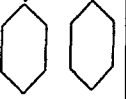
Compound A-1, β -diethylaminoethyl diphenyl acetate, may be considered the basic member of Group A. Since this study was first undertaken, other pharmacological properties of the drug, namely, antispasmodic and parasympatholytic activity, have been described. The drug is now marketed as "Trasentin" and usually little or no mention is made of its local anesthetic properties. Antispasmodic and parasympatholytic activity characterize all the members of this group of anesthetics and the relationships between chemical structure and these particular properties will be the subject of a future communication. Compound A-1 is more potent than cocaine both as a block and surface anesthetic and is less toxic. It compares favorably with many local anesthetics in clinical use. However, full anesthetic concentrations, when applied to the cornea, cause a slight degree of hyperemia.

Compound A-2 differs from A-1 only in that one phenyl group is replaced by a tolyl. This methylation increases toxicity, efficacy as a block anesthetic, and irritant properties.

Compound A-3 differs from A-1 in being an ester of benzilic rather than diphenylacetic acid. The presence of the hydroxy group decreases surface anesthetic potency and increases toxicity threefold. This particular benzilic acid derivative has many interesting pharmacological actions. It compares favorably with papaverine as an antispasmodic and approaches atropine in parasympatholytic activity. Its varied pharmacological actions will form the basis of a separate report.

Compound A-4 incorporates the changes occurring in A-2 and A-3. The high

TABLE 1

COM- POUND	STRUCTURAL FORMULA	RABBIT CORNEA			FROG UROSTYLE			M.L.D. ₅₀ (MOUSE INTRA- PERIT.)	INDEX OF ANESTHETIC POTENCY		TOXIC- ITY INDEX	THERAPEUTIC RATIO	
		Mini- mal anesthet- ic dose per cent	Dura- tion of anes- thesia (after 2 per cent)	Irritation	Mini- mal anesthet- ic dose	Dura- tion M.A.D.	Dose active for 180 min.		Cor- nea	Uro- style		Cor- nea	Uro- style
Co- caine	$ \begin{array}{c} \text{CH}_3\text{OOC}\cdot\text{HC}-\text{CH}-\text{CH}_3 \\ \quad \\ \text{COO}\cdot\text{HC} \quad \text{N}\cdot\text{CH}_3 \\ \quad \\ \text{H}_3\text{C}-\text{CH}-\text{CH}_3 \end{array} $ 	0.5	20	+	0.04	30	0.18	90	1.0	1.0	1.0	1.0	1.0
A-1	$ \begin{array}{c} \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \\ \diagdown \quad \diagup \\ \text{CHCOOCH}_2\text{CH}_2\text{N} \\ \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \end{array} $ 	0.25	21	+	0.02	40	0.04	300	2.0	2.0	0.3	6.6	6.6
A-2	$ \begin{array}{c} \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \\ \diagdown \quad \diagup \\ \text{CHCOOCH}_2\text{CH}_2\text{N} \\ \quad \\ \text{CH}_3 \quad \text{C}_6\text{H}_5 \end{array} $ 	0.25	40	++	0.015	20	0.03	225	2.0	2.7	0.4	5.0	6.7
A-3	$ \begin{array}{c} \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \\ \diagdown \quad \diagup \\ \text{COHCOOCH}_2\text{CH}_2\text{N} \\ \quad \\ \text{C}_6\text{H}_5 \quad \text{C}_6\text{H}_5 \end{array} $ 	0.5	18	+	0.02	60	0.035	100	1.0	2.0	0.9	1.1	2.2

Hydrolyzed in solution

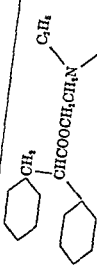
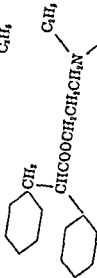
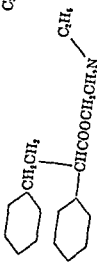
	0.25	30	+	0.03	45	0.045	100	2.0	1.3	0.9	2.2	1.4
4												
5		1.0	20	+	0.05	180	200	0.5	0.8	0.45	1.1	1.8
6												
7		1.0	20	+	0.04	30	0.03	0.25	0.5	0.14	3.6	7.1
8		0.25	13	+	0.02	60	0.04	75	2.0	1.2	1.7	1.7
9		0.25	44	+	0.035	30	0.045	150	2.0	1.1	3.3	1.8

TABLE 1—Concluded

COM- POUND	STRUCTURAL FORMULA	RABBIT CORNEA			FROG UROSTYLE			M.L.D. ₁₀₀ (MOUSE INTRA- PERIT.)	INDEX OF ANESTHETIC POTENCY		TOXIC- ITY INDEX	THERAPEUTIC RATIO	
		Mini- mal anest- hetic dose	Dura- tion anest- hesia (after 2 per cent)	Irritation	Mini- mal anest- hetic dose	Dura- tion M.A.D.	Dose active for 180 min.		Cor- nea	Uro- style		Cor- nea	Uro- style
A-10		0.125	62	+	0.01	180	0.011	75	4.0	4.0	1.2	3.3	3.3
A-11		1.0	52	+	0.01	30	0.015	150	0.5	4.0	0.6	0.8	6.7
A-12		0.25	22	++	0.015	45	0.035	250	2.0	2.7	0.36	5.6	7.5
A-13		0.25	43	++	0.015	120	0.035	150	2.0	2.7	0.6	3.3	4.5

NEW LOCAL ANESTHETICS

33 4.5

A-14		1.0	23	++++	?	?	?	200	0.5	?	0.45	1.1	?
A-15		0.25	37	++++	?	?	?	150	2.0	?	0.6	3.3	?
A-16		0.25	25	++++	?	?	?	250	2.0	?	0.36	5.6	?

block anesthetic than the foregoing members of the series, but is equal in potency to A-1 as a surface anesthetic.

Compound A-5, the methoxy derivative of A-1, is less potent as an anesthetic and has sufficient toxicity so that its therapeutic ratio is low.

Compound A-6, the dibutylamino homologue of A-1, undergoes spontaneous hydrolysis in solution with a consequent loss of activity.

Compound A-7 features a substitution of morpholine for the diethylamine of A-1. It is the least toxic of all the compounds studied and still retains a degree of activity equal to that of cocaine as a blocking agent.

Compound A-8 is representative of the familiar procedure of lengthening the ester chain in synthetic local anesthetics, being the propyl ester homologue of A-1. Toxicity is markedly increased but no change occurs in anesthetic potency, a striking example of the dissociation of anesthetic and toxic properties.

Compound A-9 is also a propyl ester but differs from A-8 in that the tertiary amine is substituted on the beta carbon. Both toxicity and activity as a blocking agent are reduced.

Compound A-10 incorporates multiple changes in that it is the γ -dibutylaminopropyl ester of benzoic acid. When compared with the diethylaminoethyl ester of benzoic acid (A-3), it is found to be very much more potent, with respect both to minimal anesthetic concentrations and duration of action, and is only slightly more toxic. The increase in activity can be ascribed to the presence of the dibutyl amine. The high toxicity cannot be attributed solely to the benzoate structure inasmuch as the change from an ethyl to a propyl ester in the diphenyl acetate compounds (A-1 vs. A-8) results in a product that is as toxic as A-10. Unfortunately, dibutylaminopropyldiphenyl acetate was not available for study. The high activity of A-10 prompted a study of its intravenous toxicity in rabbits. The fatal dose is 5.0 mgm. per kilogram as compared with 15.0 mgm. per kilogram for cocaine. The discrepancy between the toxicity index after intraperitoneal injection in mice (1.2) and after intravenous administration in rabbits (3.0) is explicable on the basis of a more rapid rate of destruction of this type of synthetic ester than cocaine.

Compound A-11 is unique in this series, being a β -diethylaminoethyl ester of diphenyleneglycolic acid. When the anesthetic is compared with A-3, it is seen that the change to the diphenylene structure has resulted in a decrease in toxicity, a decrease in potency but an increase in duration of action as a surface anesthetic, and an increase in potency as a block anesthetic.

Compound A-12 differs from compound A-1 only in being a propionate rather than an acetate. The outstanding effect is to increase the irritant property. There is also a slight increase in toxicity and activity as a block anesthetic.

Compound A-13 differs from A-12 in being a propyl ester. Toxicity and duration of action are thereby considerably increased without significant change in minimal anesthetic concentrations. A similar increase in toxicity results in the acetate series in the change from an ethyl to propyl ester (A-1 vs. A-8).

Compound A-14 differs from A-1 in being a phenylbenzyl acetate rather than a diphenyl acetate. This change is markedly detrimental, resulting in an ex-

trely irritating compound with greater toxicity and less anesthetic potency than the parent substance.

Compound A-15 in the propyl ester series differs from A-8 in the same manner that A-14 in the ethyl ester series differs from A-1, namely in being a phenylbenzyl rather than a diphenyl compound. Irritation is again greatly increased. In this instance, however, toxicity is reduced and anesthetic potency is unaltered. This indicates that no general conclusion can be drawn with regard to the effects of the same substitution in two series of compounds as closely related as the ethyl and propyl esters. Compound A-15 must also be compared with A-14 from which it differs only in being a propyl rather than an ethyl ester. This change has increased anesthetic potency and toxicity.

Compound A-16 differs from A-15 in that the benzyl group is replaced by a phenylethyl. The anesthetic and irritating properties are not altered, but the toxicity is somewhat decreased.

GROUP B. The compounds of group B, except for B-8, consist of phenylalkyl or alkylene derivatives of acetic acid alkamine esters. The quantitative pharmacological data on these compounds are presented in table 2.

Compound B-1 is β -diethylaminoethylphenylethylidene acetate. The replacement of one phenyl of A-1 by a two carbon unsaturated chain destroys anesthetic activity.

Compound B-2, β -diethylaminoethylphenylallyl acetate has regained anesthetic potency, but when compared with A-1 it is much weaker as a surface anesthetic.

Compound B-3 differs from B-2 in being a propyl rather than an ethyl ester. This change doubles activity as a surface anesthetic and diminishes toxicity, the latter change being the opposite of that noted in the A group upon replacing an ethyl with a propyl ester.

Compound B-4, the butylphenyl acetate, has regained the full activity of the diphenyl compounds. Toxicity and irritant property, however, are greatly increased.

Compound B-5 differs from B-4 in being the propyl rather than the ethyl ester. This change has resulted in a decrease in toxicity and irritant property without altering surface anesthetic potency. Again in this series, the change from the ethyl to propyl ester is accompanied by a decrease in toxicity.

Compounds B-6 and B-7, both amylphenyl acetates, are too hygroscopic to permit quantitative work.

Compound B-8, a triethyl acetate, is without anesthetic activity.

GROUP C. Group C consists of four symmetrical alkamine esters of diphenylsuccinic acid. The pharmacological data for this series are presented in table 3.

Compound C-1, the simplest member of the group, is symmetrical β -diethylaminoethyldiphenyl succinate. When compared with β -diethylaminoethyl-diphenyl acetate, it is less potent both as a surface and block anesthetic and approximately as toxic.

Compound C-2, the symmetrical dibutylamino homologue of C-1, is inactive, probably as the result of spontaneous hydrolysis.

Compound C-3, which differs from C-1 in being the propyl rather than the ethyl ester, is somewhat more toxic, no more potent as a surface anesthetic, and slightly less potent as a blocking agent. An outstanding property of the com-

TABLE 2

COM- POUND	STRUCTURAL FORMULA	RABBIT CORNEA			M.L.D. ₁₀₀ (MOUSE INTRA- PERIT.)	INDEX OF ANES- THETIC POTENCY CORNEA	TOXIC- ITY INDEX	THERA- PEUTIC RATIO CORNEA
		Mini- mal anes- thetic dose	Dura- tion an- esthesia (after 2 per cent)	Irrita- tion				
		per cent	minutes		mgm./ kg.			
B-1		Inactive						
B-2		2.0	6	+	300	0.25	0.3	0.6
B-3		1.0	7	+	600	0.5	0.15	3.3
B-4		0.25	24	+++	100	2.0	0.0	2.2
B-5		0.25	24	+	150	2.0	0.6	3.3
B-6		Hygroscopic						
B-7		Hygroscopic						
B-8		Inactive						




pound, however, is its long duration of anesthesia, evident in regard both to the cornea and the spinal cord.

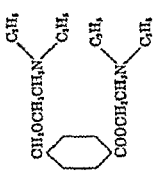
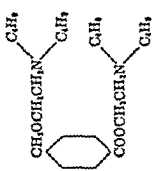
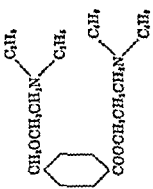
Compound C-4 is the symmetrical dibutylamino homologue of C-3. The

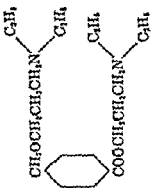
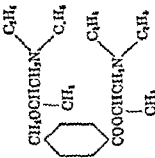
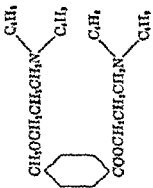
TABLE 3

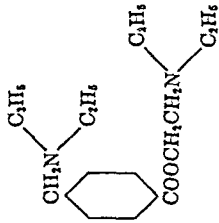
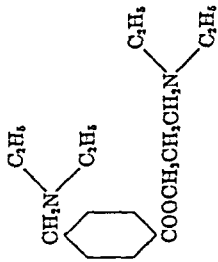
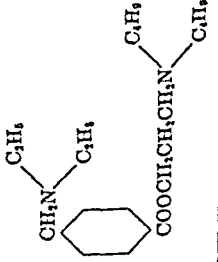
COM- POUND	STRUCTURAL FORMULA	BAREIT CORNEA		2% UROSTYLE			M.L.D. 100 (MOUSE INTRA- PERIT.)	INDEX OF ANESTHETIC POTENCY		TOX- ICITY INDEX	THERAPEUTIC RATIO		
		Min- imal anest- hetic dose 2 per cent	Dura- tion after 2 per cent	Irrita- tion	Min- imal anest- hetic dose	Dura- tion M.A.D.		Dose active for 100 min.	Cornea		Uro- style	Cornea	Uro- style
Inactive (Hydrolysis)													
C-1		2.0	9	+	0.03	45	0.07	300	0.25	1.3	0.3	0.8	4.3
C-2													
C-3		2.0	65	+	0.04	130	0.045	200	0.25	1.0	0.45	0.55	2.2
C-4		2.0	30	+++	0.05	120	0.055	400	0.25	0.8	0.22	1.1	3.6

TABLE 4

COM- POUND	STRUCTURAL FORMULA	RABBIT CORNEA			MINIMAL ANESTHETIC DOSE UROSTYLE mgm./gram	M.L.D. ₅₀ (DOSE PER TRAFERIN) mgm./kg	INDEX OF ANES- THETIC POTENCY		TOXICITY INDEX	THERAPEUTIC RATIO	
		Minimal anesthetic dose per cent	Duration anesthesia (after 2 per cent)	Irrita- tion			Cornea	Uro- style		Cornea	Uro- style
D-1	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_2\text{OCH}_2\text{CH}_2\text{N} \\ \quad \\ \text{C}_2\text{H}_5 \end{array}$ 	2.0	11	+	0.06	100	0.25	0.67	0.9	0.28	0.74
D-2	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_2\text{OCH}_2\text{CH}_2\text{N} \\ \quad \\ \text{C}_2\text{H}_5 \end{array}$  COOC_2H_5	0.75	15	+	0.06	300	0.67	0.67	0.3	2.2	2.2
D-3	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_2\text{OCH}_2\text{CH}_2\text{N} \\ \quad \\ \text{C}_2\text{H}_5 \end{array}$  COOC_2H_5	0.5	27	+	0.03	400	1.0	1.3	0.22	4.5	5.9

		Inactive									
		2.0	25	+	0.06	400	0.25	0.67	0.22	1.1	3.0
D-4											
D-5											
D-6		0.5	14	+	0.02	200	1.0	2.0	0.45	2.2	4.4

D-10		Inactive		0.1	?	—	0.4	?	—	?	
D-11		Inactive									
D-12		0.37	35	+++	0.03	300	1.35	1.3	0.3	4.5	4.3

COM- POUND	STRUCTURAL FORMULA	RABBIT CORNEA			MINIMAL ANESTHETIC DOSE UROSTYLE mgm./gram	M.L.D. ₁₀₀ (HOUSE IN- TRAPERIT.) mgm./kg	INDEX OF ANES- THETIC POTENCY		TOXICITY INDEX	THERAPEUTIC RATIO	
		Minimal anesthetic dose per cent	Duration anesthesia (after 2 per cent)	Irrita- tion			Cornea	Uro- style		Cornea	Uro- style
D-13											
D-14											
D-15											
		2.0	9	+	?	400	0.25	?	0.22	1.1	?

Inactive

main result of this change is a decrease in toxicity and an increase in irritant property. A unique feature of this compound is the fact that the onset of corneal anesthesia is delayed for seven minutes after topical application.

GROUP D. The compounds of this group consist of derivatives of benzyl ethers and benzylamines. Their pharmacological data are presented in table 4.

Compound D-1, β -diethylaminoethylbenzyl ether, may be considered the parent substance of the benzyl ether derivatives. In itself, it is a highly toxic anesthetic of low activity.

Compound D-2 is the *p*-ethyl carboxylate of D-1. This change has markedly increased the potency of the compound as a surface anesthetic and decreased the toxicity.

Compound D-3, the *p*-butyl carboxylate of D-1, is even more potent both as a surface and block anesthetic, and considerably less toxic. Thus not only does the ester group enhance the activity and decrease the toxicity of the alkamine ether, but increasing the length of the ester chain further increases anesthetic potency and decreases toxicity.

Compound D-4 differs from D-2 in that the simple ethyl ester has been converted to a diethylaminoethyl ester, the ether portion of the molecule remaining unchanged. The resultant compound, 4-diethylaminoethylcarboxylate of diethylaminoethylbenzyl ether, has lost all anesthetic activity. Thus the change from ethylcarboxylate to diethylaminoethylcarboxylate is detrimental and it would appear that *p*-alkamine esters are less effective than simple alkyl esters of benzyl ethers.

Compound D-5 is 4-dibutylaminoethylcarboxylate of dibutylaminoethylbenzyl ether. As a result of changing both the ester and ether alkamines from diethyl to dibutyl, minimal anesthetic activity is restored, associated with low toxicity.


Compound D-6 is similar to D-4 in the ether portion of the molecule, but the ester group has been changed from diethylaminoethyl to dibutylaminopropyl. This change in the ester portion of the molecule not only restores activity, but results in a fairly potent compound. Thus, *para* substitution of alkamine esters which are sufficiently long-chained can potentiate the anesthetic activity of alkamine benzyl ethers.

Compound D-7 can best be compared to D-2, the only change being from an ethyl to a propyl ether. Anesthetic activity is adversely affected and toxicity is unchanged by increasing the length of the ether chain.

Compound D-8 differs from D-7 in being a dibutylamino rather than a diethylaminopropylbenzyl ether. The change from the diethyl to dibutyl amine increases anesthetic activity and decreases toxicity. However, the irritant property of the compound has been increased.

Compound D-9 is 4-diethylaminoethylcarboxylate of diethylaminopropylbenzyl ether. Its inactivity might be ascribed either to the detrimental effect of the propyl ether, as judged from the fact that previously this change (D-7 vs. D-2) was detrimental to activity; or to the fact that the ester chain (diethylaminoethyl) is too short to bring out the anesthetic activity of the ether (see D-4); or to both factors.

TABLE 5

IND EX	R	N	R	-R-	-O-		-COO-	-R-	R	CORNEA MINIMAL ANESTHETIC DOSE	UROSTYLE MINIMAL ANES- THETIC DOSE	M.L.D. ₁₀₀ (MOUSE INTRAPERITONEAL)
										per cent	mgm./gram	mgm./kg
0	C ₂ H ₅			C ₂ H ₅	+		-	C ₂ H ₅	C ₂ H ₅	2.0	0.06	100
1	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	0.75	0.06	300
2	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	0.5	0.03	400
3	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	—	—
4	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	2.0	0.06	400
5	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	0.5	0.02	200
6	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	1.5	0.07	300
7	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	1.0	0.04	400
8	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	—	—
9	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	0.1	?
10	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	—	—
11	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	0.37	0.03	300
12	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	—	—
13	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	—	—
14	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	—	—	—
15	C ₂ H ₅			C ₂ H ₅	+		+	C ₂ H ₅	C ₂ H ₅	2.0	?	400

Compound D-10 is 4-diethylaminopropylcarboxylate of diethylaminopropylbenzyl ether. One feature of this compound has previously been shown to be associated with a decrease in activity, namely, the propyl ether. Inasmuch as activity would seem to be returning, as evidenced by the slight action of the compound as a block anesthetic, this must be ascribed to the fact that it is a propyl ester.

Compound D-11 differs from *D-10* only in having isopropyl rather than propyl groups in the ester and ether portions of the molecule. The slight activity as a block anesthetic, noted in *D-10*, has disappeared as a result of this change.

Compound D-12, 4-dibutylaminopropylcarboxylate of dibutylaminopropylbenzyl ether, has a higher molecular weight than any member of the series. One feature of this compound, namely, the propyl ether, has previously been shown to be detrimental to anesthetic activity. Consequently the high potency must be attributed to the dibutylamino groups in both the ether and ester portions of the molecule as well as to the propyl ester. Although the compound has a relatively low toxicity, it is highly irritating.

Compounds D-13, D-14 and D-15 comprise three derivatives of diethylbenzylamine. *D-13*, 4-diethylaminoethylcarboxylate of diethylbenzylamine, and *D-14*, 4-diethylaminopropylcarboxylate of diethylbenzylamine are both inactive as anesthetics. However, compound *D-15*, 4-dibutylaminopropylcarboxylate of diethylbenzylamine, has minimal anesthetic activity associated with low toxicity. Obviously, dialkylaminobenzyl ethers are more potent anesthetics than benzylalkyl amines.

Group D as a whole shows most interesting relationships between chemical structure and pharmacological activity. These are depicted in table 5.

SUMMARY

Forty-three compounds were studied for their local anesthetic properties, toxicity and relationship between chemical structure and pharmacological action. They consist of (A) alkamine esters of diphenylacetic acid, diphenylpropionic acid, benzilic acid and closely allied derivatives; (B) alkamine esters of phenyl alkyl and phenyl alkylene substituted acetic acid; (C) symmetrical alkamine esters of diphenylsuccinic acid; (D) *para* alkyl and alkamine carboxylates of alkamine benzyl ethers and diethyl benzylamine. All the groups contain highly active members.

(1) All members of group A are active. In general, increasing the length of the ester or alkamine groups increases toxicity and anesthetic potency, but there are several notable exceptions.

(2) Phenyl alkylene acetates are inactive if the alkylene group contains only two carbon atoms, but activity appears with a three carbon chain. Phenyl butyl acetates are as active as diphenyl acetates.

(3) Alkamine esters of diphenylsuccinic acid are characterized by relatively high thresholds of anesthetic activity but exceedingly long durations of action.

(4) Diethylaminoethylbenzyl ether has weak local anesthetic activity. *Para* substitution of ethyl and butyl carboxylate progressively potentiates

anesthetic activity and decreases toxicity. Activity is lost if the *p*-ethyl carboxylate is converted to the *p*-diethylaminoethyl carboxylate and is only regained by increasing the length of the ester or alkamine chain. Lengthening the ether from ethyl to propyl diminishes activity whereas increasing the alkamine portion of the ether chain from diethyl to dibutyl enhances potency. Para alkamine carboxylates of diethylbenzylamine are active only after the carboxylate has been increased in molecular weight to the dibutylaminopropyl ester.

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THE EFFECT OF SYMPATHOMIMETIC AMINES ON PANCREATIC SECRETION

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The effect of various substances other than secretin on the external secretion of the pancreas following their intravenous injection has been the subject of considerable investigation, as a result of which it is now believed that certain compounds stimulate and others inhibit. In the former category are peptones (1), pilocarpine (2), physostigmine (3), prostigmine (4), muscarine (5), trimethylamine (6), quaternary ammonium salts (7), choline (8), atropine in large doses (9), histamine (10), methylguanidine (11), barium chloride (12), and nitrites (13). The substances which inhibit include atropine (14), epinephrine (15), and ephedrine (16). In general, it may be stated that compounds which possess a cholinergic action might be expected to cause the pancreas to secrete, due to stimulation of the vagus secretory fibers to the gland; and those which are vasodilators stimulate secretion as a result of increased blood flow through the pancreas, the secretion of which has been shown to be affected by changes in vascular tone (17, 18). Conversely, atropine is stated (14) to inhibit pancreatic flow by eliminating that component of secretion arising from vagus activity, and epinephrine and ephedrine probably operate through their vasoconstrictor effect (15, 16).

The present experiments are the outgrowth of a study of the effect of miscellaneous drugs on pancreatic secretion and gall-bladder tone and motility. In the course of this work it was noted that epinine, one of the substances tested, stimulated pancreatic secretion, and that certain characteristics differentiated this stimulation from that evoked by secretin. This observation appeared particularly striking because of the obvious structural similarity (see table 1) between epinine and epinephrine, an established inhibitor of pancreatic secretion. Consequently a large series of sympathomimetic amines has been tested with a view to ascertaining which of them possess the property manifested by epinine, and, if possible, the molecular structure necessary for such action. At the same time, comparative quantitative studies were made of the inhibitory effect on pancreatic secretion evidenced by the drugs of this series.

EXPERIMENTAL. Intact dogs of various sizes were anesthetized with sodium pentobarbital, the abdomen opened by a midline incision, and the main pancreatic duct cannulated. A record was made of carotid blood pressure, and of pancreatic flow by means of an electric drop counter, and a small dose of a potent secretin concentrate (SI) was injected to provide assurance that the animal's pancreas was properly responsive. The sympathomimetic amines to be tested were then injected in varying quantities, the dosage depending on the degree of blood pressure rise and the presence or absence of toxic manifestations.

In order to determine inhibition of secretion produced by the drugs the animals were

given a continuous intravenous injection of a dilute secretin solution through a Woodyatt pump at a rate of approximately 0.1 mgm. per minute. When the secretory rate became constant at a rate of 1 to 2 drops per minute the drug under investigation was injected and the alteration in secretion observed. To place these findings on a quantitative basis the animal's blood pressure response to a known amount of epinephrine (0.1 or 0.01 mgm.) was determined, together with the extent and duration of pancreatic inhibition produced thereby. Subsequently the dosage of the unknown drug requisite to produce approximately the same rise in blood pressure was ascertained by a series of trials, and the degree of pancreatic inhibition produced by the epinephrine equivalent was noted.

RESULTS. A. *Sympathomimetic amines stimulating pancreatic secretion.* Six of the compounds tested were potent to a greater or lesser degree in causing the pancreas to secrete when injected intravenously in a dosage of 1 to 10 mgm. These were, in the order of their effectiveness, hydroxytyramine, epinine, *m*-hydroxyphenethylamine, *m*-methoxyphenethylamine, *p*-methoxyphenethylamine, and 3,4-dimethoxyphenethylamine. The pressor action was most pronounced in the case of epinine and least so in the methoxy compounds. When cocaine was injected in a dosage of 3 mgm. per kilo prior to the administration of these drugs, there was a marked potentiation of both pressor action and pancreatic stimulation. Ergotamine in 3-5 mgm. doses diminished or abolished the pressor response to these compounds, but left undiminished the secretory response of the pancreas. An illustrative record is reproduced in figure 1.

B. *Sympathomimetic amines inhibiting pancreatic secretion.* The great majority of the compounds tested fell into this category. When the "epinephrine equivalent" of the drug examined was injected intravenously into a dog whose pancreas was caused to secrete at a constant rate, there was a retardation in pancreatic flow of varying duration, usually coextensive with the pressor response. In some instances the inhibition was followed by a brief secondary acceleration over the control rate.

The detailed data for all the drugs tested, including the pressor epinephrine equivalents, are listed in table 1.

DISCUSSION. An examination of the data reveals that the majority of the sympathomimetic amines tested exerted an inhibitory effect on pancreatic secretion. This undoubtedly is due directly to their vasoconstrictor action whereby the blood supply to the pancreas is decreased and consequently the circulating secretin is prevented free access to the gland. Although crucial evidence in this regard is lacking, since there is no satisfactory method of measuring blood flow through the pancreas, there is an abundance of presumptive support. Plethysmographic measurements (44) on the pancreas reveal significant changes in size of the gland following vagus stimulation indicative of increased blood supply; and it has been noted by one of us (45) that generalized active hyperemia induced by artificial hyperpyrexia results in an increased responsiveness by the pancreas to secretin stimulation, whereas artificially depressing the body temperature has a converse effect. The other possible mechanism whereby secretion may be depressed is by a constriction in the duct system of the pancreas, a circumstance which has also been demonstrated to occur following vagus stimulation (46). In this event the initial inhibition should be followed by a marked secondary stimu-

lation. Such a situation was found to obtain in the case of some of the compounds tested (table 1), so that with these particular drugs this mechanism may have been operative partially—not completely, since the secondary stimulation was in no instance marked or prolonged, nor did it compensate for the deficiency existing during the phase of inhibition.

Three of the substances tested—oxytyramine, epinine, and *m*-hydroxyphenethylamine—occupy a unique position in this series of compounds, in that they stimulate the pancreas to secrete instead of inhibiting it. Four others—*m*-methoxy- and *p*-methoxy-phenethylamine, 3,4-dimethoxyphenethylamine, and 3,4-dihydroxyphenylalanine are also stimulators, though to a considerably lesser degree. The mechanism whereby this takes place is obscure; however, certain characteristics sharply distinguish it from secretin stimulation. In the first place the secretory response to the drug is completely abolished by atropine, whereas that to secretin is not; secondly, secretin fails to cause the pancreas to secrete after ergotamine, which, however, does not diminish the response to the sympathomimetics. There are three possible mechanisms by which these drugs

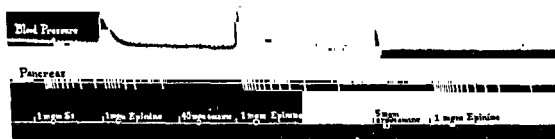


FIG. 1. PANCREATIC RESPONSE IN DROPS TO SECRETIN AND TO EPININE BEFORE AND AFTER COCAINE AND ERGOTAMINE

may act. They may (a) produce a local vasodilatation in the pancreas; (b) they may stimulate secretory nerves which are probably vagal in derivation, since sympathetic secretory fibers to the pancreas have never been demonstrated; or (c) they may act directly on the acinar cells. The present demonstration that these drugs can stimulate a secreting gland is not an entirely new departure; it was previously shown by one of us (47) that epinine has the property of stimulating gastric secretion. Furthermore, it is well known that adrenergic drugs stimulate the salivary glands to form a sparse, thick secretion (48).

Regarding the molecular structure necessary for the effect noted, a considerable degree of specificity appears to exist. Thus phenethylamine and tyramine fail to stimulate, but a shift of the hydroxyl group on the ring to the 3-position relative to the side chain results in a stimulator, and addition of an hydroxyl group to form a 3,4-dihydroxyphenyl structure results in a marked increase in potency. Monomethylation of the amino group results in only a slight decrease in effectiveness, but dimethylation completely abolishes it and results in an inhibitor. Likewise, an inhibitor is obtained when the carbon atom in the α -position to the

TABLE 1

Pressor and pancreatic secretory effects of sympathomimetic amines


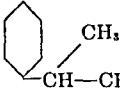






COMPOUND	FORMULA	EPINEPHRINE EQUIVALENT	PANCREATIC INHIBITION	
			Extent <i>per cent</i>	Duration <i>min.</i>
Phenethyl amine (19, 20)	 <chem>CCNCC1=CC=CC=C1</chem>	4×10^{-3} (b)	75	1.5
Phenyl-2-amino-1-propane (21)	 <chem>CNC(C)CC1=CC=CC=C1</chem>	2×10^{-3} (b)	68	5
N-methylphenethylamine (22, 20)	 <chem>CNC(C)CC1=CC=CC=C1</chem>	9.4×10^{-3} (a)	60*	2
Pervitin (23, 24) desoxyephedrine	 <chem>CNC(C)CC1=CC=CC=C1</chem>	1.9×10^{-4} (b)	60	2.5
Phenyl-3-amino-1-propane (25, 20)	 <chem>NCCCC1=CC=CC=C1</chem>	2.3×10^{-3} (b)	60	2
Propadrine (26, 27)	 <chem>CNC(C)C(O)C1=CC=CC=C1</chem>	1×10^{-2} (a)	17*	1
d-Ephedrine (28, 29)	 <chem>CNC(C)C(O)C1=CC=CC=C1</chem>	1.5×10^{-3} (b)	50	1½
Ephetonal (30)	 <chem>CNC(C)C(O)C1=CC=CC=C1</chem>	4×10^{-3} (b)	100	2½

TABLE 1—Continued









COMPOUND	FORMULA	EPINEPHRINE EQUIVALENT	PANCREATIC INHIBITION	
			Extent <i>per cent</i>	Duration <i>min.</i>
Tyramine (31, 20)	 <chem>CC(N)Cc1ccc(O)cc1</chem>	4.7×10^{-3} (b)	67*	2½
M-hydroxyphen- ethylamine (20)	 <chem>CC(N)Cc1ccc(O)cc1</chem>	6.6×10^{-3} (b)	Stimulates	
Paredrinol, Veritol (32)	 <chem>CCNC(C)Cc1ccc(O)cc1</chem>	1×10^{-3} (a)	40	5
Synephrine Sympa- thol (33)	 <chem>CCNC(C)Cc1ccc(O)cc1</chem>	9.5×10^{-4} (b)	33	1
Oxytyramine (34, 20)	 <chem>CC(N)Cc1ccc(O)cc1O</chem>	5×10^{-3} (b)	Stimulates	
Epinine (35, 20)	 <chem>CCNC(C)Cc1ccc(O)cc1O</chem>	3×10^{-3} (a)	Stimulates	
Arterenol (36, 37)	 <chem>CC(N)Cc1ccc(O)cc1O</chem>	3×10^{-3} (a)	76*	4
Epinephrine (38, 39)	 <chem>CCNC(C)Cc1ccc(O)cc1O</chem>	1	50† 67†	4† 10†

TABLE 1—Continued

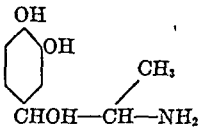
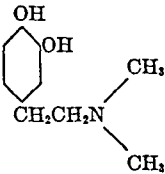
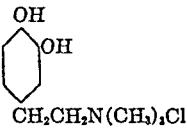
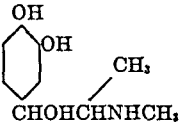
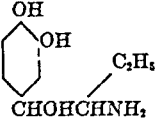
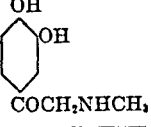
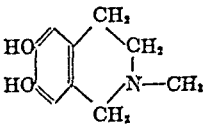



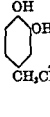
COMPOUND	FORMULA	EPINEPHRINE EQUIVALENT	PANCREATIC INHIBITION	
			Extent per cent	Duration min.
Corbasil, cobefrine (40, 41)	 <chem>CC(C1CCC(O)C1O)CNC</chem>	2.5×10^{-1} (a)	57*	3
3,4-Dioxyphenethyl- dimethylamine (21)	 <chem>CN(C)CCc1ccc(O)c(O)c1</chem>	1.3×10^{-2} (a)	67	9
Corynein chloride (21, 41)	 <chem>CCN(C)CCc1ccc(O)c(O)c1.Cl</chem>	2×10^{-1} (a)	83*	4
Dioxyephedrine (41)	 <chem>CC(C1CCC(O)C1O)NC</chem>	3.8×10^{-2} (a)	87	10
Ethyl nor-suprarenin (41)	 <chem>CCNC(C1CCC(O)C1O)C</chem>	1.9×10^{-2} (b)	62	6
Adrenalone, kephrine (42, 20)	 <chem>CCNC(=O)C1CCC(O)C1O</chem>	3.6×10^{-2} (b)	78	7
6,7-Dihydroxytetra- hydro-2-methyl- isoquinoline (35)	 <chem>CC1CN(C)CCc2cc(O)c(O)cc21</chem>	2×10^{-2} (b)	33	5

TABLE 1—*Concluded*

COMPOUND	FORMULA	EPINEPHRINE EQUIVALENT	PANCREATIC INHIBITION	
			Extent <i>per cent</i>	Duration <i>min.</i>
<i>p</i> -Methoxyphenethyl- amine (43)		5×10^{-4} (b)	Stimulates	
<i>m</i> -Methoxy-phenethyl- amine (43)		4.5×10^{-4} (b)	Stimulates	
3,4-Dimethoxyphen- ethylamine (43)		0	Stimulates	
3,4-Dioxyphenylal- anine (44)		0	Stimulates	

(a) Standardized to 0.1 mgm. epinephrine.

(b) Standardized to 0.01 mgm. epinephrine.

* Secondary stimulation.

† 0.01 mgm.

‡ 0.1 mgm.

ring is substituted by an hydroxyl or a keto-group. Methylation of the phenolic hydroxyl groups results in a decrease, but not a complete loss, in potency; but peculiarly, when this procedure is applied to tyramine, an inhibitor is transformed into a stimulator. A schematic representation of the structural modifications discussed above is shown in figure 2.

A considerable number of other related compounds remain to be evaluated before it can be determined to what extent the potency is altered by modifications in structure. This we are confident of accomplishing, since we have at hand, in the remarkable secretion-stimulating property of these materials, a biological test which is most reliable. At present the following requirements have been established:

- (1) An hydroxyl group on the ring in the 3-position to the sidechain; two such groups in the 3,4-positions increase the potency markedly.
- (2) An unsubstituted carbon atom in the α -position to the ring.
- (3) A primary or secondary amino nitrogen on the side chain.

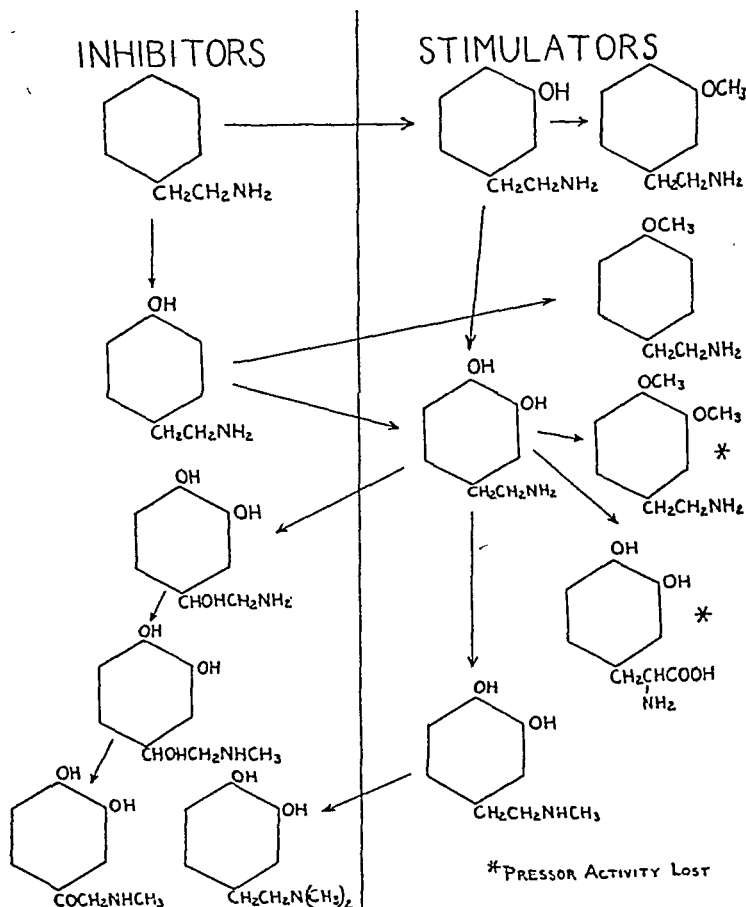


FIG. 2

The epinephrine equivalents listed in table 1 represent the results obtained on a series of several dogs for each compound. In most cases they are at variance with those recorded in the literature.

SUMMARY

A series of 27 sympathomimetic amines has been tested for their effect on pancreatic secretion. Twenty-one of these compounds inhibited the secretion evoked by continuous secretin stimulation. The mechanism whereby this took

place is probably on a vasoconstrictor basis. Seven of the compounds stimulated pancreatic secretion; these were all structurally similar to the extent of hydroxylation of the benzene ring in specific positions, an unsubstituted carbon atom in juxtaposition to the ring, and a primary or secondary amino nitrogen. Methylation of the hydroxyl groups leaves the secretory potency attenuated but not abolished. The type of stimulation evidenced by these compounds differs from secretin stimulation.

Acknowledgment

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HEMODYNAMIC EFFECTS OF INTRAVENOUS MORPHINE AND PENTOTHAL SODIUM

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Therapeutic doses of morphine administered subcutaneously to man and animals have been found to produce no significant cardiovascular changes except bradycardia (8, 10). Conflicting reports regarding the circulatory adjustments in response to intravenous thiobarbiturates such as pentothal sodium in normal animals and man appear in the literature (4, 6, 7). However, it is generally agreed that pentothal sodium reduces the blood pressure in arterial hypertension. The investigation herein reported was undertaken to secure further experimental data on the hemodynamic effects of morphine sulphate and pentothal sodium given intravenously to normal man.

METHODS. Volunteer normal convalescent patients from the Third (N. Y. U.) Medical Division of Bellevue Hospital were studied at least two hours post prandial after 30 to 60 minutes of bed rest. Morphine sulphate (0.01 gm. in 5 cc. normal saline) was injected rapidly into the cubital vein following control observations in six subjects. Pentothal sodium (5 per cent aqueous solution) was given intravenously on three occasions to unpremedicated subjects (0.5 gm. in 53 sec., 1.0 gm. in 65 sec., 1.0 gm. in 30 sec.).

Cardiac output was determined by the ballistocardiographic method, using the area equation of Starr *et al.* (9). The ballistocardiograph used in this work was developed by Dr. Dugald Brown and has been described elsewhere (2). The values obtained have been corrected by a factor derived from the work of Cournand and Ranges (3) where a constant discrepancy between cardiac output as determined by the direct Fick method and the ballistocardiograph was demonstrated. The Hamilton optical manometer was used to measure arterial pressure directly, mean pressure being determined by planimetric integration of the area under the pulse pressure curve (5). The brachial artery was the take-up point for arterial pressure when pentothal sodium was used, while the radial was employed during the morphine studies. Using the formula derived from Frank's air-chamber theory of hemodynamics (see Aperia (1) for derivation), peripheral vascular resistance was calculated in absolute units, dynes cm.⁻² sec. $\left(R = \frac{Pm \times 1332}{c.o.} \right)$ where R is peripheral resistance, Pm mean pressure and $c.o.$ cardiac output per second.)

RESULTS. I. Morphine. When morphine sulphate was given intravenously, the patients always became drowsy and occasionally fell asleep. This sedative action appeared approximately 10 minutes after injection and by 20 minutes had passed its peak. The only subjective effect was transient head pain which appeared immediately after administration.

Study of table 1 reveals no essential change in cardiac output immediately

¹ Commonwealth Fund Fellow.

(within $1\frac{1}{2}$ minutes) after giving the drug except in one case where a rise in cardiac output of 20 per cent was observed. There were no delayed effects on the cardiac output in five of the six cases, but one showed an increase of 20 per cent. The change in pulse rate was insignificant. Two of the four subjects upon whom blood pressure measurements were made showed little change in systolic, diastolic or mean arterial pressures. There was a rise in all pressures immediately after injection of the drug in one case and in one other there was a delayed rise in blood pressure. Associated with these changes in blood pressure were parallel changes in peripheral resistance. The changes in peripheral resistance ranged from -18 per cent to +28 per cent; it rose in two subjects and fell in two others.

II. Sodium pentothal. Following the administration of pentothal sodium, there was immediate loss of consciousness in all instances. One subject (V.) remained in light surgical anesthesia (first plane) for four minutes after 0.5 gm. of pentothal sodium was injected over a period of 53 seconds. The second subject was given 1.0 gm. more slowly (65 seconds) and after $1\frac{1}{2}$ minutes was anesthetized to second plane surgical anesthesia for a period of 9 minutes. The third subject was anesthetized profoundly to respiratory arrest almost immediately after the injection of 1.0 gm. of the drug in 30 seconds. From a hemodynamic standpoint the first two subjects may be considered together and the last described separately.

The output of the heart remained unchanged throughout the period of observation in the first two cases. The stroke volume showed a tendency to decrease, with a compensatory increase in pulse rate, while the blood pressure fell. The change in peripheral resistance was not significant. The changes in these two normal subjects (An. and V.) are summarized in table 1.

The third subject exhibited marked clinical and circulatory changes. There was immediate respiratory arrest lasting almost 2 minutes, followed by 30 minutes of deep third plane anesthesia complicated by asphyxia and excessive secretion of mucus. Within 20 seconds of administration of the drug, there was a marked elevation of peripheral resistance accompanied by a parallel increase in systolic, diastolic and mean pressures. At this time there was no significant change in cardiac output. It is interesting to note that a relative bradycardia occurred only during the first 10 seconds after the injection of the drug. At 50 seconds the blood pressure fell, the systolic pressure falling below control values and the diastolic and mean pressures to the control level. Two minutes after the drug was given asphyxia supervened, with increased cardiac output attributable to tachycardia rather than increase in stroke volume, and this was followed shortly by hypertension. There was also a reduction in peripheral resistance. At 4 and 7 minutes a marked pulsus alternans appeared on the arteriogram and ballistocardiogram. Observations were not carried beyond 10 minutes. These asphyxial effects were so severe that repetition of this injection (1.0 gm. in 30 seconds) was deemed unsafe.

DISCUSSION. Morphine has been shown to exert its usual clinical effects when

TABLE 1

In the following table each control datum represents an average of two or more observations. The average of two to five observations made during the first 90 seconds following the administration of the drug was used for the figures listed under immediate effects. The data included under delayed effects represent the average of 3 to 10 observations made after 90 seconds over a period of 10 to 60 minutes.

SUBJECT	TIME	PULSE RATE	STROKE VOLUME	CARDIAC INDEX	BLOOD PRESSURE	MEAN ARTERIAL PRESSURE	PERIPHERAL RESISTANCE
<i>Morphine</i>							
H.	Control	70	64.6	2.58			
	Immediate effect	65	63.5	2.36	-9		
	Delayed effect	63	68.3	2.44	-5		
A.	Control	80	65.5	3.38			
	Immediate effect	87	63.2	3.53	+4		
	Delayed effect	73	64.3	3.10	-8		
O.	Control	60	70.3	2.43	123/68	89	1789
	Immediate effect	70	67.8	2.72	+11 128/70	97	1645
	Delayed effect	67	75.8	2.92	+20 111/65	86	1355 -18
Mc.L.	Control	90	74.7	3.97	152/79	105	1249
	Immediate effect	90	69.4	3.70	-7		
	Delayed effect	83	77.7	3.81	-4 173/105	128	1598 +27
P.	Control	95	79.9	3.79	150/74	110	1170
	Immediate effect	100	91.2	4.56	+20 162/68	111	976 -16
	Delayed effect	100	81.3	4.05	+7 163/69	106	1052 -10
S.	Control	76	77.6	3.33	117/55	76	1009
	Immediate effect	83	70.7	3.32	0 137/69	9	1299 +28
	Delayed effect	64	83.4	3.06	-8 116/58	79	1188 +17

Pentothal Sodium

V.	Control	90	79.3	4.05	112/58	74	825	
	Immediate effect	100	74.1	4.21	+4 89/55	66	712	-14
	Delayed effect	94	72.5	3.88	-4 95/55	67	789	-5
An.	Control	64	90.8	3.05	116/34	58	749	
	Immediate effect	75	82.4	3.24	+6 110/32	54	745	0
	Delayed effect	69	77.4	2.82	-8 96/36	54	808	8
Pe.	Control	93	72.2	4.23	157/76	109	1300	
	8 sec	78	77.5	3.83	-9 162/83	118	1542	+19
	20 sec.	97	65.1	3.46	-8 181/103	145	1845	+42
	50 sec.	117	61.7	4.56	+8 138/81	107	1264	-3
	1' 12"	107	69.7	4.68	+11 134/78	98	1105	-15
	2' 15"	113	73.7	53.1	+26 146/91	113	1084	-17
	*4'	125	64.6	5.11	+21 168/108	129	1169	-10
	4' 50"	137	72.6	6.23	+47 151/97	120	976	-25
	*6' 25"	137	71.2	6.12	+45 167/98	129	1051	-10
	7' 50"	127	77.0	6.33	+50 173/101	127	1000	-22
	9' 15"	112	82.9	5.82	+38 154/93	117	1041	-20

given intravenously, without producing any consistent circulatory change. The hemodynamic changes following morphine were unpredictable in direction and, even though significant, were not of considerable magnitude. The use of the drug intravenously in therapeutic doses, has been found to be safe during the course of this study.

Light surgical anesthesia produced by the intravenous administration of pentothal sodium (5 per cent) seemed to have little effect upon the circulation of normal man. However, the dose and rate of injection required for light anesthesia were difficult to predict on any given occasion. The rate of injection of the drug is of great importance in determining its effects. Large doses and rapid rates of administration have been used in all cases. It must be emphasized, however, that as much as 1.0 gm. of the drug given in one minute has no essential hemodynamic effect, whereas the same dose given twice as fast, as suggested for clinical use by Guedel, may have pronounced circulatory effects (3).

The response to the rapid administration of a large dose discloses a biphasic action which is frequently seen in the effects of drugs on the central nervous system. The initial bradycardia and the rapid rise in peripheral resistance and blood pressure were possibly the result of excitation of the vasomotor center. All these effects occurred in less than 30 seconds and were immediately followed by complete reversal, i.e., by a decrease in blood pressure and peripheral resistance, with no significant change in cardiac output. This is possibly attributable to peripheral vasodilatation. The picture after the first two minutes was complicated by asphyxia, as evidenced by cyanosis. At this time there was a rise in blood pressure despite the decrease in peripheral resistance and the pulse rate and cardiac output rose rapidly.

SUMMARY

1. Circulatory studies of the effect of morphine and pentothal sodium given intravenously in normal man are described.

2. Morphine intravenously in therapeutic doses (10 mgm.) has no significantly consistent effect on the cardiovascular system (stroke volume, heart rate, blood pressure and peripheral resistance).

3. Pentothal sodium intravenously, in doses of 0.5 gm. given in 53 seconds and 1.0 gm. in 65 seconds, has little circulatory effect in normal man. In a single instance, a dose of 1.0 gm. given intravenously over a period of 30 seconds produced transient respiratory arrest. Marked vasoconstriction and bradycardia occurred at first, followed rapidly by vasodilatation, with no significant change in cardiac output before asphyxial changes supervened.

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THE QUANTITATIVE NATURE OF THE COACTION OF BISMUTH AND ARSENICAL COMPOUNDS IN THE THERAPY OF EXPERIMENTAL SYPHILIS

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Soon after the introduction of arsphenamine by Ehrlich as a chemotherapeutic agent in the treatment of syphilis, it was realized that the drug was too toxic to make possible the cure of the disease with one large dose, and it became necessary to give repeated small doses over a long period of time to give satisfactory clinical results. Subsequently, it was found that better results were obtained when mercury was administered as an adjunct to the arsphenamine. After the introduction of bismuth by Levaditi and his associates in 1921, its superiority to mercury as an adjunct to the arsenical drugs soon became recognized, and it has all but eliminated the use of mercury in the present day treatment of syphilis.

Numerous studies have appeared to prove decisively that the results of treatment, expressed especially in terms of ultimate clinical outcome, are better if arsenical and bismuth compounds are both employed than if an arsenical compound is used alone. However, with the exception of a preliminary report from this laboratory (1), there is a definite lack of information concerning the quantitative nature of the coaction of bismuth and arsenical compounds in the therapy of syphilis, i.e., whether or not they are additive, potentiative or inhibitive. Pharmacologically, this information is of theoretical interest as well as of practical importance. The explanation for this lack of information is due to the relative difficulty of determining minimal curative and maximal tolerated doses of the drugs in human beings. Such data are necessary if one is to study therapeutic and toxic coaction of drugs in a quantitative way. The present report deals with the experimental investigation of this problem in the rabbit.

METHODS. *Compounds employed.* Mapharsen and neoarsphenamine were the arsenical compounds chosen because we had accumulated considerable data previously which became of value in the preliminary experiments. After the investigation was well under way we found that neoarsphenamine, when given in combination, did not give the sharp and consistent results obtained with mapharsen. Consequently, it was decided to use the latter as the arsenical drug for the remainder of the combination therapeutic studies. Our preliminary report (1) was on the combination of mapharsen and neoarsphenamine, respectively, with bismuth sodium tartrate. The question arose whether other bismuth compounds behaved as did bismuth sodium tartrate when given in combination with mapharsen. Instead of testing all of the many bismuth compounds used in the therapy of syphilis, it seemed justifiable to select representative types differing in solubility and rates of absorption from intramuscular depots. A comparative study of various bismuth compounds (2, 3, 4) gave the necessary preliminary information of importance for the

selection of these types. The following three compounds were selected:¹ thiobismol, a water-soluble preparation, very rapidly absorbed; bismuth sodium tartrate, a water-soluble preparation, quite readily absorbed, and bismuth ethyl camphorate, an oil-soluble preparation, rather slowly absorbed.

Combination therapy of rabbit syphilis. Healthy male rabbits weighing approximately 2 kilograms were inoculated with the Nichols' strain of *Treponema pallidum*. This strain has been maintained in our laboratory since 1923 by passage through rabbits at intervals of six to eight weeks. The inoculations were made into the testes using an inoculum prepared in the usual manner from fresh chancre material from an infected rabbit and of such concentration that 0.5 cc. of this suspension when injected caused lesions to appear in 5 to 6 weeks. Blood Wassermann tests were made at the Wisconsin Psychiatric Institute, under the direction of Dr. W. F. Lorenz, using a test especially adapted to the serological reactions of the rabbit. In all of our therapeutic experiments the following procedure was employed. Only rabbits with typical lesions were chosen on the sixth to eighth week after being infected. In order to determine the minimal curative dose of a compound, rabbits were treated with three weekly injections of each compound. The minimal curative dose (M.C.D.) was that dose which when given three times at weekly intervals cured 66 per cent

TABLE 1

Summary of the minimal curative doses in rabbit syphilis of the various compounds employed

COMPOUND	MINIMAL CURATIVE DOSE		ROUTE OF ADMINISTRATION
	mgm./kgm.	mgm. Bi/kgm.*	
Mapharsen	1.0		Intravenous
Neocarsphenamine.	10.0		Intravenous
Bismuth sodium tartrate		1.0	Intramuscular
Bismuth ethyl camphorate.. . . .		2.0	Intramuscular
Thiobismol		1.5	Intramuscular

* All doses of bismuth preparations, both toxic and therapeutic, referred to hereafter, were calculated on the basis of milligrams of bismuth as the metal per kilogram of body weight and will be expressed as mgm. Bi/kgm.

of the animals. As a criterion of cure, popliteal lymph nodes from each treated animal were transferred, three weeks after the last treatment, into the testes of each of two recipient animals. Only rabbits with negative Wassermann reactions were used, and these were then observed for at least 8 to 12 weeks for the development of lesions or positive serology.

From previous studies we had data available on the M.C.D. of mapharsen, neocarsphenamine, bismuth sodium tartrate, bismuth ethyl camphorate, and thiobismol (table 1). The M.C.D. for each compound was rechecked before this study was undertaken.

The method employed for determining the nature of the coaction of bismuth and arsenical compounds was to treat infected animals at the usual time with combinations of the fractional portions of the M.C.D. of one or the other of each type of compound. The fractions of the M.C.D. of each drug were given three times at intervals of one week. The bismuth preparation employed was given intramuscularly the first part of the week and the arsenical drug was given intravenously two to four days later. From preliminary studies, better coaction seemed to occur if the arsenical was given a few days after the administration of the bismuth preparation, thus permitting sufficient time for the bismuth to reach its maximum

¹ We wish to thank Parke, Davis & Company for the mapharsen and thiobismol, G. D. Searle & Company for the bismuth sodium tartrate, and The Upjohn Company for the bismuth ethyl camphorate used in this work.

of absorption and thereby allowing the two compounds to act together at their peak of concentration in the body. It was found that better therapeutic results were obtained when the arsenical drug was given two days after the administration of the more rapidly absorbed thiobismol and three to four days after the more slowly absorbed tartrate and camphorate preparations.

Certain terms have been used to describe the various ways in which drugs may act in combination. It will perhaps be appropriate here to define these terms. When the action of two drugs administered at the same time is equal to the sum of their individual effects, the response is termed *simple addition*. For example, if 25, 50 or 75 per cent of the M.C.D. of an arsenical be given with 75, 50 or 25 per cent, respectively, of the M.C.D. of a bismuth preparation and cure is obtained with each combination, simple addition has occurred. We

TABLE 2

Summary of combination therapy with mapharsen and bismuth sodium tartrate (intramuscularly)

PERCENTAGE OF THE M.C.D. OF BISMUTH SODIUM TARTRATE	PERCENTAGE OF THE M.C.D. OF MAPHARSEN	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS CURED	NUMBER OF ANIMALS NOT CURED
25	50	3	0	3
50	25	4	2	2
50	50	3	3	0
25	75	4	4	0
75	25	4	4	0

TABLE 3

Summary of combination therapy with nearsphenamine and bismuth sodium tartrate (intramuscularly)

PERCENTAGE OF THE M.C.D. OF BISMUTH SODIUM TARTRATE	PERCENTAGE OF THE M.C.D. OF NEO- ARSPHENAMINE	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS CURED	NUMBER OF ANIMALS NOT CURED
25	25	4	0	4
25	50	5	2	3
50	25	2	1	1
50	50	7	2	5
25	75	4	4	0
75	25	4	1	3

can then say that these compounds are therapeutically *additive*. If the effects should be less than the sum of their individual effects, i.e., if the above combinations of fractions fail to cure, the response will be termed *antagonism* or *inhibition*. On the contrary, if fractions of each drug totaling less than 100 per cent of the M.C.D. of both drugs be used, e.g., 25 per cent of the M.C.D. of a bismuth compound and 50 per cent of the M.C.D. of an arsenical, and cure be obtained, the effects are greater than the sum of their individual effects and the response will be termed *potentiation*. It is with this interpretation of terminology that we shall present the results of our combination therapeutic studies.

THERAPEUTIC RESULTS. By using combinations of the fractions of the M.C.D. of an arsenical drug with fractions of the M.C.D. of a bismuth preparation, we were able to determine the quantitative nature of the combined thera-

peutic action of these two types of compounds. It was found that it was one of simple addition rather than potentiation or inhibition (tables 2, 3, 4, 5 and 6).

Results obtained with mapharsen and bismuth sodium tartrate (table 2) are especially uniform. Only 2 out of 7 animals treated with combinations totaling less than 100 per cent of the M.C.D. of both compounds were cured, indicating a lack of potentiation of one compound by the other. Using various combinations of each compound totaling 100 per cent of the M.C.D., of eleven animals treated, 100 per cent were cured. This indicates quite conclusively that addition has occurred and also that there is no inhibition of one compound by the other.

TABLE 4

Summary of combination therapy with mapharsen and bismuth ethyl camphorate (intramuscularly)

PERCENTAGE OF THE M.C.D. OF BISMUTH ETHYL CAMPHORATE	PERCENTAGE OF THE M.C.D. OF MAPHARSEN	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS CURED	NUMBER OF ANIMALS NOT CURED
25	50	1	1	0
50	25	2	0	2
50	50	5	3	2
25	75	3	3	0
75	25	7	4	3

TABLE 5

Summary of combination therapy with mapharsen and thiobismol (intramuscularly)

PERCENTAGE OF THE M.C.D. OF THIOBISMOL	PERCENTAGE OF THE M.C.D. OF MAPHARSEN	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS CURED	NUMBER OF ANIMALS NOT CURED
25	50	2	0	2
50	25	2	2	0
50	50	4	3	1
25	75	3	3	0
75	25	6	1	5

With combinations of neoarsphenamine and bismuth sodium tartrate (table 3), the results are not as uniform as with mapharsen but, nevertheless, the coactions of these two compounds also are quite obviously of an additive nature. The inconstancy of the results with neoarsphenamine was not unexpected. We cannot attribute this to differences in lots of the drug since the same was used throughout this as well as the preliminary study.² Because of the inconsistent results obtained with neoarsphenamine in this type of study, we decided to abandon its use in further studies, as mentioned above, and to use only mapharsen as the arsenical drug.

Tables 4 and 5 represent the results obtained when mapharsen was combined

² We have found from previous experience that the determination of the M.C.D. or of single minimal curative doses of the same lot, or different lots, of neoarsphenamine varies considerably over given periods of time. Unlike neoarsphenamine, mapharsen is constant in this respect.

with bismuth ethyl camphorate and thiobismol, respectively. From these data the evidence is quite conclusive that the coaction between either of these two bismuth compounds and mapharsen is also simple addition.

Upon careful examination of the different combinations of doses in tables 2, 3, 4 and 5, it is quite manifest that a certain combination of fractions of the M.C.D. of each type of compound seems to produce better addition than others. We refer particularly to the combination of 25 per cent of the M.C.D. of any of the bismuth preparations with 75 per cent of the M.C.D. of the arsenical. In the case of each combination at these dosages, all of the animals were cured, while with the reverse combination of fractions, i.e., 75 per cent of the M.C.D. of a bismuth preparation with 25 per cent of the M.C.D. of an arsenical, the addition was not so complete in all instances, with the exception of the combination of mapharsen and bismuth sodium tartrate in which 100 per cent of the animals were cured. This finding we are unable to explain. In general, however, in most of the combinations totaling 100 per cent of the M.C.D. of both types of compounds, over 66 per cent of the animals treated were cured. There-

TABLE 6

Summary of combination therapy with mapharsen and bismuth sodium tartrate (intravenously)

PERCENTAGE OF THE M.C.D. OF BISMUTH SODIUM TARTRATE	PERCENTAGE OF THE M.C.D. OF MAPHARSEN	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS CURED	NUMBER OF ANIMALS NOT CURED
25	50	3	3	0
50	25	1	0	1
50	50	3	3	0
25	75	2	2	0
75	25	2	1	1

fore, it seems justifiable to conclude from the evidence presented that, therapeutically in rabbit syphilis, the quantitative nature of the coaction of bismuth and arsenical drugs is that of simple addition.

We had considerable data available on the treatment of rabbit syphilis with bismuth compounds administered intravenously; consequently, it seemed of interest to ascertain whether or not mapharsen combined with bismuth would be additive when both were administered by this route. Bismuth sodium tartrate was chosen as the bismuth preparation. The M.C.D. for this drug when given intravenously was found to be 1.5 mgm. per kgm. (3). The same combination of fractions of the M.C.D. of bismuth sodium tartrate and of mapharsen were used in this experiment as were used in the previous experiments. Results were essentially the same as those obtained when bismuth sodium tartrate was given intramuscularly (table 6).

COMBINATION TOXICITY IN RABBITS. In the evaluation of any drug or drugs to be employed in chemotherapy, our main objective should be to obtain as wide a margin of safety as possible. This holds for drugs given in combination as well as for those given alone. Because of the paucity of specific information on

the combination toxicity of these two types of compounds, it was necessary to include in this study a determination of this point in order to establish a basis for their margins of safety. We were interested primarily in the question of whether or not the combined toxicity was more or less than the summation of the respective maximal tolerated doses of each compound. If the cototoxicity³ was found to be less than additive, i.e., if more than 50 per cent of the maximal tolerated dose (M.T.D.) of each compound could be given, a greater margin of safety would be available than if either compound were given alone up to its maximum of tolerance. On the contrary, if the cototoxicity was found to be more than additive, i.e., if less than 50 per cent of the M.T.D. of each compound could be tolerated, there would be a decrease in the margin of safety. The margin of safety would then be less than if either compound were given alone up to its maximum of tolerance.

Healthy rabbits of about 2 kgm. body weight were used in this study. They were kept under uniform housing conditions and fed on a diet of alfalfa and oats. Combinations of doses from 50 to 100 per cent of the respective M.T.D.₆₆ of an

TABLE 7

Summary of the maximal tolerated doses in rabbits of the various compounds employed

COMPOUND	MAXIMAL TOLERATED DOSE		ROUTE OF ADMINISTRATION
	mgm./kgm	mgm. Bi/kgm	
Mapharsen	10		Intravenous
Neosarsphenamine	150		Intravenous
Bismuth sodium tartrate		59	Intramuscular
Bismuth sodium tartrate		4	Intravenous
Bismuth ethyl camphorate		40	Intramuscular
Thiobismol		2	Intramuscular

arsenical drug with the various types of bismuth preparations were employed. The same compounds were used in the toxicity as in the therapeutic studies. The single M.T.D. of each compound is listed in table 7. The dose at which 66 per cent of the animals survived was chosen as the M.T.D.₆₆.

In the preliminary toxicity study of each combination of drugs, the bismuth compound was given first, followed by the arsenical either on the same day or one, two or three days later. The purpose of administering the arsenical drug at various intervals after the bismuth preparations was to allow ample time for the bismuth compounds to attain their peak concentration in the susceptible tissues, in order that the two types of drugs be permitted to act when they were both at their peak concentrations. However, in these preliminary experiments, it did not seem to make any difference in the combined M.T.D.₆₆ when the arsenical was given. As a matter of convenience, therefore, they were injected at the same treatment period in the cototoxicity experiments. The doses at which 66 per cent of the animals survived was chosen as the combined M.T.D.₆₆.

³ For brevity, we shall use the term "cototoxicity" to mean combination toxicity of bismuth and arsenical compounds administered concurrently.

COTOXICITY RESULTS. With combinations of mapharsen and neoarsphenamine, respectively, with bismuth sodium tartrate (tables 8, 9, 10), combina-

TABLE 8

Summary of combination toxicity with mapharsen and bismuth sodium tartrate (intramuscularly)

PERCENTAGE OF THE M.T.D. OF MAPHARSEN	PERCENTAGE OF THE M.T.D. OF BISMUTH SODIUM TARTRATE	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS LIVED	NUMBER OF ANIMALS DIED
60	60	3	2	1
60	70	3	3	0
70	60	3	2	1
70	70	3	2	1
60	80	3	1	2
80	60	3	1	2
70	80	3	0	3
80	70	3	0	3
80	80	3	0	3
80	90	1	0	1
90	80	2	1	1

TABLE 9

Summary of combination toxicity with mapharsen and bismuth sodium tartrate (intravenously)

PERCENTAGE OF THE M.T.D. OF MAPHARSEN	PERCENTAGE OF THE M.T.D. OF BISMUTH SODIUM TARTRATE	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS LIVED	NUMBER OF ANIMALS DIED
50	50	2	2	0
60	60	2	1	1
70	70	5	3	2
80	80	2	0	2

TABLE 10

Summary of combination toxicity with neoarsphenamine and bismuth sodium tartrate (intramuscularly)

PERCENTAGE OF THE M.T.D. OF NEO- ARSPHENAMINE	PERCENTAGE OF THE M.T.D. OF BISMUTH SODIUM TARTRATE	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS LIVED	NUMBER OF ANIMALS DIED
67	67	5	5	0
70	80	4	2	2
80	70	3	1	2
75	75	3	0	3
80	80	3	0	3
80	90	2	0	2

tions up to 70 per cent of the M.T.D.₆₈ of each compound were tolerated. Where one reckons the M.T.D.₆₈ of bismuth sodium tartrate by each route of administration, it seems to make no difference whether it be given intramuscularly or

intravenously (tables 8 and 9). We conclude, therefore, that the combination toxicity of these drugs is less than additive.

With mapharsen and thiobismol (table 11), the combined toxicity was somewhat more than with the tartrate, but still somewhat less than additive. Combination of mapharsen and thiobismol is very difficult to make, and the irregularity in the cotoxicity data with mapharsen and thiobismol is probably due to this characteristic of the latter drug.

TABLE 11

Summary of combination toxicity with mapharsen and thiobismol (intramuscularly)

PERCENTAGE OF THE M.T.D. OF MAPHARSEN	PERCENTAGE OF THE M.T.D. OF THIOBISMOL	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS LIVED	NUMBER OF ANIMALS DIED
70	70	8	4	4
75	75	5	2	3
80	70	3	2	1
70	80	3	0	3

TABLE 12

PERCENTAGE OF THE M.T.D. OF MAPHARSEN	PERCENTAGE OF M.T.D. OF BISMUTH ETHYL CAMPHORATE	TOTAL NUMBER OF ANIMALS USED	NUMBER OF ANIMALS LIVED	NUMBER OF ANIMALS DIED
70	70	3	3	0
70	80	5	4	1
80	70	4	4	0
80	80	2	2	0
80	90	1	0	1
90	80	1	1	0
90	90	2	2	0
100	100	3	0	3

When mapharsen and bismuth ethyl camphorate were combined (table 12), combinations up to 90 per cent of the M.T.D.₆₆ of each compound were tolerated. Employing combinations from 70 to 90 per cent of the M.T.D.₆₆ of each compound, only 2 out of 16 animals died. The additive effect was much less with the camphorate and mapharsen than with the other two bismuth compounds and mapharsen. Apparently the more slowly a bismuth compound is absorbed the less additive is its toxicity when combined with an arsenical drug.

DISCUSSION. In all of the combination therapeutic experiments a total of 94 syphilitic rabbits were treated, and in the combination toxicity experiments a total of 101 rabbits were employed. Mapharsen seemed to give better therapeutic coaction with bismuth than neoarsphenamine, but this does not appear of special comparative significance in the dose ranges which were used. As for the

efficacy of the various bismuth compounds when therapeutically combined with an arsenical, all seemed to be similar in their quantitative nature of coaction, i.e., all were additive. This fact is added evidence to our previous concept (2, 3) that all bismuth compounds act in a similar manner on spirochetes.

In general, the nature of the cotoxicity of bismuth and arsenical compounds is less than additive. As regards the comparison of the cotoxicities of the various bismuth compounds with mapharsen, it seems quite evident that the cotoxicity of mapharsen with a more slowly absorbed bismuth compound is much less additive than with a more rapidly absorbed bismuth preparation.

It has not been feasible to express the therapeutic efficiency of the combination of bismuth and arsenical compounds in the treatment of experimental rabbit syphilis in terms of a therapeutic index. We have been unable to conform to the laws which govern the calculation of this index because of our inability to express the therapeutic coaction and cotoxicity of the two types of drugs in terms of minimal curative and maximal tolerated doses, respectively. Thus, from the data presented, our only interpretation is that the therapeutic coaction of arsenical and bismuth compounds is that of simple addition, but that their cotoxicity is less than additive. In other words, there appears to be a greater margin of safety when bismuth and arsenical compounds are combined in the therapy of syphilis than when either is used alone in correspondingly effective doses. The combination of mapharsen with a slowly absorbed bismuth preparation seems to give the widest margin of safety, since more of each drug can be administered together than if a more rapidly absorbed type of bismuth preparation were used.

On the basis of the premises presented herein, it would seem entirely justifiable to propose the administration of relatively large doses of bismuth concurrently with large doses of the arsenical within a period of a few days, thus modifying the technique advocated by Hyman, Chargin and Leifer (5). By the use of bismuth a corresponding reduction of the arsenical drug should be adequate, and such a reduction might very notably reduce the hazards of "massive dose" therapy due to the arsenical drug.

SUMMARY AND CONCLUSIONS

By using combinations of fractions of the minimum curative dose (M.C.D.) of an arsenical drug with fractions of the M.C.D. of a bismuth preparation, we were able to determine the quantitative nature of the combined therapeutic action of these two types of compounds. It was found that it was one of simple addition rather than potentiation or inhibition.

Therapeutic addition occurs between the compounds whether the bismuth be given intramuscularly or intravenously.

All of the bismuth preparations employed in this study, namely bismuth sodium tartrate, thiobismol and bismuth ethyl camphorate, when combined with mapharsen, were similar in their quantitative nature of therapeutic coaction, i.e., all were additive.

The cotoxicity of bismuth and arsenical compounds was found to be less than

additive. It appears that the cotoxicity of an arsenical with a more slowly absorbed bismuth compound is much less additive than with a preparation more rapidly absorbed.

Since the therapeutic coactions of bismuth and arsenical compounds are completely additive while their cotoxicities are less than additive, there results a greater margin of safety when they are used concurrently than when either is used alone in correspondingly effective doses. The significance of this finding is discussed.

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THE ACTION OF PAPAVERINE ON THE HEART OF THE DOG¹

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Papaverine is an alkaloid (benzylisoquinoline) derivative of opium of low toxicity and non-habit forming (1). Macht (2) found that a weak solution (0.001 per cent) slowed the rate and caused more powerful contractions of the excised perfused frog's heart; in stronger solutions (0.1 per cent) it had no stimulating action but led to a greater slowing and a dilatation of the heart; and in still stronger solutions it occasioned further bradycardia, led to heart block and diastolic standstill. Similar effects were also observed in the cat's heart. A decrease in heart size followed 5 mgm. doses in the dog and cat, but larger doses (7.5 mgm.) caused cardiac dilatation. These actions appeared to be directly on the heart, not mediated by its extrinsic nerves. Hanzlik (3) found a prompt decrease in size of the frog and turtle heart associated with slowing and decreased contraction when the drug was used in low and medium concentrations. Complete heart standstill occurred with higher concentrations. Hale (4), on the other hand, observed an increase in cardiac rate and output with weak solutions. Snyder and Andrus (5) noted an abolition of tonus waves and an augmentation of the magnitude of contraction in the perfused turtle heart. Increased irritability leading to ventricular fibrillation in 52 per cent of the cases was observed by Gruber and Robinson (6) in the turtle heart. The fibrillation could be reversed temporarily by quinidine or morphine.

We have shown that papaverine hydrochloride is not only a powerful coronary vasodilator (7), but also that it decreases considerably the ease with which ventricular fibrillation is induced in the dog by faradic stimulation (8). Furthermore, in the presence of this drug, vigorous manual massage of the heart will restore orderly synergic beating to the fibrillating ventricles (8). The present study is concerned with a detailed examination of those effects of the drug which might throw light on its mode of action in ventricular fibrillation. The studies were directed at the effects on irritability to artificially induced auricular and ventricular stimuli, on auriculo-ventricular conductivity and on the refractory period of the ventricles. In addition, studies were made of the effect of the drug on the sinus rhythm, on the production of ectopic rhythms, and on conductivity in the various parts of the heart in anesthetized dogs.

1. EFFECT ON SINUS RHYTHM AND THE PRODUCTION OF ECTOPIC RHYTHMS AND HEART BLOCK. All experiments were made on dogs anesthetized with nembutal (25 mgm. per kilo). The observations were derived from lead II of the electrocardiogram. Papaverine was injected intravenously in every case.

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Four types of preparations were used:

(a) Animals with open chest, artificial respiration and auricles fibrillated by faradic current from a Harvard inductorium. In such experiments sinus rhythm changes could not be studied except when the auricles failed to respond to the faradic stimulation. Furthermore, no judgment could be made regarding the presence of spontaneous auricular ectopic rhythms.

(b) Animals as in (a) except that the auricles were driven at constant rate with a Lewis interrupter. In these experiments also sinus rhythm changes could not be studied.

(c) Animals as in (a) except that the heart was beating naturally.

(d) Animals with chest unopened, natural respiration and natural beating of the heart.

The details of the method of making the first two groups of preparations are given below in sections 2 and 4 of this report.

The results are summarized in table 1 which shows the frequency of the various arrhythmias in the different types of experiments. These are classified for convenience according to dosage. However, as the footnote of the table shows, there is some scattering when the dosage is calculated according to body weight. By and large, the severity of the various disturbances in rhythm fell closer into line when the results were correlated according to mgm./K. dosage.

The effect on the sinus rhythm was studied in those experiments in which the heart was beating naturally. Small doses of the drug often caused sinus acceleration which at times was marked (a rate of 250 beats per minute developed in one animal with a control rate of 100). With larger doses there were sinus slowing with sinus arrhythmia (with or without preliminary acceleration) and periods of sinus standstill of various lengths (some of which may have been actually the result of S-A block). The sinus standstills occurred more often and for longer periods as the dose of the drug was increased. During the sinus slowing and sinus standstill, nodal escapes or a nodal rhythm often appeared. In those experiments in which the auricles were stimulated faradically and the auricles failed to respond to the stimulation, the sinus rhythm which developed was also slower when larger doses of the drug were used.

With larger doses also the nodal rhythm which developed when sinus standstill was present was often slow and irregular and complete cardiac standstill sometimes ensued; this was one of the modes of death.

No auricular premature beats nor paroxysmal tachycardial attacks were encountered, but with larger doses, ventricular premature beats and paroxysmal ventricular tachycardia were frequent. The latter was at a rapid rate when it first appeared and often from multiple foci; later in the course of the experiment, however, it became slower. Sometimes the paroxysmal tachycardia was regular, more often it was irregular especially when it arose from multiple foci. The paroxysmal tachycardia usually terminated in ventricular flutter and fibrillation. Sometimes the ventricular flutter and fibrillation were paroxysmal and mingled with periods of paroxysmal ventricular tachycardia. In other instances ventricular fibrillation alternated with ventricular or heart standstill. On a few occasions the ventricular fibrillation was terminal and the cause of death. The ventricular fibrillation which appeared was of a peculiar type resembling that described by us (9) in diphtheria poisoning.

In several of the experiments in which the ventricles were stimulated electrically to induce extra responses, the beat so induced was followed by one or more premature systoles (presumably due to reentry). While this also occurred in

TABLE 1
Effect of papaverine on cardiac rhythm

	TYPE OF EXPERIMENT	DOSE OF PAPAVERINE USED	NO. OF EXPERIMENTS	SINUS ACCELERATION	SINUS SLOWING	SINUS STANOSIS	NODAL ESCAPE	NODAL RHYTHM	IDIOVENTRICULAR RHYTHM	VENTRICULAR STAND-STILL	VENTRICULAR PREMATURE SYSTOLES	VENTRICULAR PAROSTYS- MAL TACHYCARDIA	PAROSTYS- MAL TACHYCARDIA	PAROSTYS- MAL TACHYCARDIA	TERMINAL VENTRICULAR FIBRILLATION	PARTIAL A-V BLOCK	COMPLETE A-V BLOCK	INTRAVENTRICULAR BLOCK
A	Chest open, artificial respiration. Auricles stimulated with faradic current	65 mg.	5*						1		1					1	1	1
		92	5*			1	1	1			2	2				1	1	1
		222	1*					1			1	1						
		257	4*									1			1	3		3
		845	1*							1			1	1**	1			1
B	Chest open, artificial respiration. Auricles driven at constant rate artificially	27	2									1						
		92	11					1†			1†	1†						
		157	6													1		1
		260	1														2	1
C	Chest open, artificial respiration. Natural heart beat	92	6	1	4	2			1		1	2		1	1			
		287	1		1													
		325	2		2	2	2			1		2	1		1			2
D	Closed chest, natural respiration. Natural heart beat	27	2	2														
		85	2	2														
		92	4	4	2	1		1				1	1	1				1
		157	3	1	2	1	1	1				1	1	1				1
		222	2	1	2	2			2	1		2			1	1	1	1
		287	1	1	1	1			1	1				1		1	1	1

* Sinus escaped at slower than normal rate when auricles did not follow faradic current in 8 experiments.

** Restored after 41 minutes by massage but heart was then in poor condition

† Induced premature ventricular systole followed by one or more ventricular premature systoles. (This also occurred in control records.)

‡ Auricles did not follow stimulation, and nodal rhythm occurred.

§ In experiment group A.

65 mgm. of papaverine was equivalent to 3.5-5.6 mgm./K.

92 mgm., 11-18 mgm./K.

222 mgm., 22 mgm./K.

257 mgm., 21.5-45 mgm./K.

845 mgm., 84.8 mgm./K.

In experiment group B:

27 mgm., 2-2.5 mgm./K.

92 mgm., 6.6-12.3 mgm./K.

157 mgm., 9.4-15 mgm./K.

260 mgm., 16.6 mgm./K.

In experiment group C

92 mgm., 9.6-12.6 mgm./K.

257 mgm., 32 mgm./K.

325 mgm., 35-41.6 mgm./K

In experiment group D:

27 mgm., 2.7-2.9 mgm./K.

85 mgm., 5.4-6.3 mgm./K.

92 mgm., 8.6-11 mgm./K.

157 mgm., 11-13.7 mgm./K.

222 mgm., 19-22 mgm./K.

287 mgm., 32 mgm./K.

control records before papaverine was given, it was of less frequent occurrence in the absence of the drug.

Block was a common occurrence with the larger dosage of papaverine. It

consisted of intraventricular and A-V block. The former expressed itself in widening of the QRS and bizarreness of the ventricular complex; the prolongation of QRS at times was very marked. This QRS widening affected the sinus beats, the nodal beats and the beats of ventricular origin. The A-V block which developed varied from prolongation of the P-R interval, with or without occasional dropped ventricular beats, to 2:1, 3:1, 4:1 partial A-V block or complete A-V block. In the latter event, the idioventricular rhythm which appeared was slow in rate, often irregular and from multiple foci of origin. Often, too, the QRS was markedly broadened showing intraventricular block. This marked QRS widening suggests that the block was, in part at least, located in the terminal network of the conduction system since it appeared in beats originally diagnosed as arising below the bifurcation of the common bundle. Sometimes in the presence of complete A-V block, the ventricles failed to discharge and ventricular standstill, without auricular standstill, occurred.

The ectopic rhythms of the ventricles and the occurrence of A-V and intraventricular block were more frequent in the naturally beating heart than in the one with auricles artificially driven. The chief difference appears to be the maintenance of *rapid effective* heart action in the latter. This suggests that the marked cardiac slowing which occurs in the naturally beating heart with larger doses of the drug contributes to the development of block and ectopic ventricular activity. In part, this may be due to the fact that at the slower supraventricular rate ectopic pacemakers are less apt to be kept discharged. In greater part it can be attributed to the presence of cardiac ischemia, such as would occur with slow heart action, which has a tendency to aggravate the ectopic stimulating and conduction depressant action of papaverine.

The question arose as to whether these actions of papaverine were primarily on the heart or secondary to the drop in systemic blood pressure and the accompanying reduced coronary blood flow. A correlation was therefore attempted between the time of appearance of the blood pressure drop and that of the various cardiac arrhythmias in 7 experiments, 1 with 27 mgm., 1 with 65 mgm., 2 with 92 mgm., 1 with 157 mgm. and 2 with 325 mgm. of papaverine hydrochloride. It was found that in these experiments sinus slowing, sinus standstill, nodal rhythm, frequent ventricular premature systoles, varying degrees of partial A-V block and intraventricular block appeared before a significant drop in blood pressure had occurred. Furthermore the drop in blood pressure seemed to be in part the result of the cardiac slowing since the blood pressure fall progressed as the ventricular rate slowed, the two interacting in a "vicious cycle." In four experiments the drop in arterial pressure occurred simultaneously with a rise in venous pressure, confirming the opinion that the cardiac depression was at least in part responsible for the blood pressure drop. It would seem therefore that the action of papaverine in producing the arrhythmias, while aided by the blood pressure fall with its resulting cardiac ischemia, was primarily the result of a direct action on the heart.

This view was confirmed when the experiments were repeated on a "physiological heart-lung preparation." In this preparation, the open-chested anes-

thetized animal with artificial respiration has the blood supply to the body cut off and the heart pumps blood only through the lungs and the coronary vessels. This is accomplished by tying off the thoracic aorta at its descending arch, the subclavian and innominate arteries as they arise from the aortic arch, the superior and inferior vena cavae and the azygos vein. In such a preparation, the drug action can operate directly on the heart, on the pulmonary vessels, or on the coronary vessels. Nervous influences, humoral influences and actions on the peripheral systemic blood vessels are eliminated.

Four experiments were carried out with the "physiological heart-lung preparation" using 24 mgm. of papaverine hydrochloride in 2 experiments and 27 mgm. in the other two. These doses of papaverine are large for this preparation since the mass of tissue is much less than in the intact animal. In one of the experiments with 27 mgm. of papaverine, the auricle was stimulated electrically at a constant rate. In this experiment, papaverine led to a prolongation of the *P-R* interval within 8 min., intraventricular block appeared at the same time and about 2 minutes later 2:1 *A-V* block developed. In the other experiment with 27 mgm. of papaverine the heart was beating naturally. Papaverine led to a sinus slowing during the first 2 minutes, after which intermittent sinus standstill and nodal rhythm appeared. At the end of 3½ minutes a paroxysmal ventricular tachycardia occurred which caused a significant fall in blood pressure and led, in another minute, to the development of the peculiar type of ventricular fibrillation referred to above. In one of the two experiments with 24 mgm. of papaverine a sinus slowing appeared during the first minute, after which a permanent sinus standstill with a nodal rhythm developed; the rate of the latter was slightly below the previous sinus rate. The nodal rhythm continued to slow and the heart finally ceased to beat entirely. As the nodal rate slowed the blood pressure fell progressively. In the fourth experiment with 24 mgm. of papaverine, the sinus rhythm slowed progressively and became irregular during the first 6 minutes, first degree *A-V* block and intraventricular block appeared at the second minute and became progressively more marked. As the heart slowed the blood pressure dropped 30 mm. Hg during this first 6 minute interval. At the 7th minute complete sinus standstill with a slow nodal rhythm appeared, the nodal rhythm then slowed progressively, the intraventricular block became more marked and at the 10th minute the peculiar type of slow ventricular fibrillation supervened.

It is therefore apparent that since the cardiac arrhythmias described in the intact animal were reproduced for the most part in the "physiological heart-lung preparation," their occurrence is primarily the result of a direct action of papaverine on the heart. Slowing of the heart and the blood pressure drop contribute in the intact animal to the appearance of these arrhythmias but are not essential.

2. RESPONSE OF THE AURICLES TO FARADIC STIMULATION. A systematic survey was made of the responsiveness of the auricles to sustained faradic current stimulation in a total of 16 experiments, 11 innervated and 5 denervated. No difference in the results were seen between the innervated and the denervated

consisted of intraventricular and A-V block. The former expressed itself in widening of the QRS and bizarreness of the ventricular complex; the prolongation of QRS at times was very marked. This QRS widening affected the sinus beats, the nodal beats and the beats of ventricular origin. The A-V block which developed varied from prolongation of the P-R interval, with or without occasional dropped ventricular beats, to 2:1, 3:1, 4:1 partial A-V block or complete A-V block. In the latter event, the idioventricular rhythm which appeared was slow in rate, often irregular and from multiple foci of origin. Often, too, the QRS was markedly broadened showing intraventricular block. This marked QRS widening suggests that the block was, in part at least, located in the terminal network of the conduction system since it appeared in beats originally diagnosed as arising below the bifurcation of the common bundle. Sometimes in the presence of complete A-V block, the ventricles failed to discharge and ventricular standstill, without auricular standstill, occurred.

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The question arose as to whether these actions of papaverine were primarily on the heart or secondary to the drop in systemic blood pressure and the accompanying reduced coronary blood flow. A correlation was therefore attempted between the time of appearance of the blood pressure drop and that of the various cardiac arrhythmias in 7 experiments, 1 with 27 mgm., 1 with 65 mgm., 2 with 92 mgm., 1 with 157 mgm. and 2 with 325 mgm. of papaverine hydrochloride. It was found that in these experiments sinus slowing, sinus standstill, nodal rhythm, frequent ventricular premature systoles, varying degrees of partial A-V block and intraventricular block appeared before a significant drop in blood pressure had occurred. Furthermore the drop in blood pressure seemed to be in part the result of the cardiac slowing since the blood pressure fall progressed as the ventricular rate slowed, the two interacting in a "vicious cycle." In four experiments the drop in arterial pressure occurred simultaneously with a rise in venous pressure, confirming the opinion that the cardiac depression was at least in part responsible for the blood pressure drop. It would seem therefore that the action of papaverine in producing the arrhythmias, while aided by the blood pressure fall with its resulting cardiac ischemia, was primarily the result of a direct action on the heart.

This view was confirmed when the experiments were repeated on a "physiological heart-lung preparation." In this preparation, the open-chested anes-

In 4 of the 5 experiments with 65 mgm. of papaverine hydrochloride, A-V conductivity was practically unchanged, in the 5th partial and complete A-V block developed. In the 5 experiments with 92 mgm., one showed no significant change, one revealed some enhancement of A-V conductivity, two showed a temporary slight depression of A-V conductivity, and the 5th a definite depression leading to complete A-V block. The experiments with larger doses, 1 with 222 mgm., 4 with 287 mgm., and 1 with 872 mgm., all showed partial and/or complete A-V block. No change in A-V conductivity was observed in the control experiment in which 5 cc. of HCl (pH = 3.3) was used.

Thus while papaverine in small doses may on occasion augment A-V conductivity, it usually tends to depress A-V conductivity and in larger doses this may lead to complete A-V block.

That this effect is a direct one on the heart was shown by the development of A-V block with papaverine in the experiments on the "physiological heart-lung preparation" reported in section 1, as well as by the observations during the first 45 seconds after papaverine administration in the experiment on the "physiological heart-lung preparation" reported in section 2. In this last experiment, auricular fibrillation as a result of faradic stimulation continued for 45 seconds after papaverine administration, and during this time the ventricular response was found to have slowed to 165 beats/min. from a value of 210 beats/min. existing before the drug was given. There can thus be no doubt that the effect on A-V conductivity, at least its depression, is due to a direct action of papaverine on the heart.

4. THE RESPONSE OF THE VENTRICLES TO STIMULI INTRODUCED AT A FIXED POINT IN DIASTOLE. Since our earlier experiments (8) suggested that papaverine rendered the ventricles unresponsive to faradic stimulation, we attempted to determine whether or not the drug would also make the ventricles unresponsive to single electrical shocks administered in a fixed part of diastole.

For this purpose single stimuli were introduced in the ventricles at a fixed point in diastole every fifth heart cycle and the ability of the ventricles to respond was noted at various intervals after drug administration. Dogs of 20 to 40 kilos were prepared as usual except that the auricles were driven at a constant rate throughout the experiment by electrical break shocks from a Lewis interrupter, applied by means of a pair of platinum hook electrodes fastened to the tip of the right auricle. The rate was set at about 50 beats faster than the natural heart rate in order to prevent the sinus node from taking over the control, since, as stated earlier, papaverine tends to accelerate the sinus rate when given in small doses. Such a change in heart rate would invalidate the analysis since the ventricular stimuli would not occur at the same point of the heart cycle throughout the experiment. The ventricular stimuli were delivered by a second cam on the Lewis interrupter mounted on the rod carrying the auricular stimulating cam. This was set so as to furnish a break shock every fifth auricular stimulus at a fixed point in late diastole. The stimulating platinum hook electrodes were fastened to the right ventricle away from the septum in a region devoid of blood vessels. The exposed surface of the heart was kept warm by a lamp and moist with lukewarm saline. The secondary coil connected to the ventricular electrodes was set just above the threshold necessary to give a response in the control period.

The response to stimulation was determined by electrocardiographic recording and in each instance the fidelity of the technique was checked before the experiment was started.

hearts. The effect of the acidity of the papaverine hydrochloride was checked by using a 5 cc. solution of HCl ($\text{pH} = 3.3$) in one experiment as a control and the HCl was found to be without effect.

Dogs weighing 4 to 11 kilos were anesthetized with nembutal (25 mgm./K. intravenously). The heart was exposed by removing the anterior portion of the thorax and artificial respiration was instituted. In some experiments, the heart was denervated by sectioning the vagi in the neck and removing the upper 6 thoracic sympathetic and the stellate ganglia. The auricles were stimulated with a faradic current of minimal strength from a Harvard inductorium in order to induce auricular fibrillation. The stimulating electrodes consisted of platinum hooks attached to the tip of the right auricle through a pericardial slit. Appropriate doses of papaverine hydrochloride were injected intravenously. Records were taken at one-half minute intervals for the first 5 minutes after injection of the drug and then at about 7, 10, 15, and, on occasion, 30 minutes after the drug was given. The records were compared with the control tracing taken before the drug was administered.

In 2 experiments, 65 mgm. of papaverine hydrochloride had no significant effect on the auricular response to the faradic current; in the other 3, however, the auricular response was slowed and, at times, the auricles became irresponsive and sinus rhythm reappeared. In 2 of the 5 experiments with 92 mgm. of the drug, no significant effect on the auricular response occurred; in the other 3, however, the auricular rate became slower indicating fewer responses to the faradic stimuli, and in two of these (the ones with the largest dose calculated as mgm./K.) the auricles became irresponsive for a time to the faradic current, the sinus node resuming control. In larger doses, 1 with 227 mgm., 4 with 287 mgm., and 1 with 872 mgm., papaverine abolished the responsiveness of the auricles to the faradic current for varying periods of time, the sinus node assuming control instead.

Thus it can be seen that papaverine tends to depress the responsiveness of the auricles to faradic stimulation. In some instances this depression was great enough to abolish the response entirely. In this last respect the auricles behaved in the same manner as the ventricles in earlier studies (8).

That this effect is a direct one on the heart was shown by repeating the experiment in the "physiological heart-lung preparation" described in the preceding section. In one experiment 24 mgm. of papaverine hydrochloride was given intravenously; within 45 seconds the auricles failed to respond to the faradic stimulation, and during the next 10 minutes this continued to be the case even though the secondary coil of the inductorium was advanced upon the primary as far as possible. There can therefore be no doubt that the depression of responsiveness of the auricles by papaverine is a direct effect on the heart.

3. *A-V CONDUCTIVITY.* The preceding experiments were also utilized to determine *A-V* conductivity according to the technique previously described by us (10). The *A-V* conductivity was measured by the ventricular rate determined while auricular fibrillation was present, using the number of *QRS* complexes occurring in 4 second intervals as the measure. Those experiments in which no auricular fibrillation could be induced after the drug, or in which the auricular rate was slowed considerably by it, were discarded for this analysis unless noticeable *A-V* block developed.

istered, the ventricles fibrillated. At this point, the interruptor was stopped and 45 seconds later the ventricular fibrillation spontaneously ceased. When the stimulator was started again, 7 minutes after the injection of the drug, the ventricles were found to respond to only about 60 per cent of the ventricular stimuli.

It appears, therefore, that papaverine in doses of 92 mgm. to 157 mgm. diminishes the irritability of the ventricles rendering them less responsive to extrinsic stimuli. This probably is the mode by which the preventive action of the drug operates experimentally. If a similar action occurs as regards spontaneous ectopic foci in the ventricles, a simple explanation of the "antifibrillatory action" is at hand. That this unresponsiveness of the ventricles is due to a direct action on the heart was tested in the "physiological heart-lung preparation" described in section 1. In one experiment, 27 mgm. of papaverine hydrochloride given intravenously caused a state of unresponsiveness of the ventricles beginning at the 8th minute so that during the next 3 minutes no responses were obtained. At the end of this time (11 minutes after papaverine administration) the current strength was increased and it was found that a fourfold increase was required before the stimuli became effective. There is thus no doubt that the depression of irritability of the ventricles following papaverine is due to a direct effect on the heart.

5. THE REFRACTORY PERIOD OF THE VENTRICLES. The decrease in irritability of the ventricles shown in the preceding experiments and in our earlier work with faradic stimulation (8) suggests the possibility that papaverine alters the refractory period. The decrease in the number of auricular responses to faradic stimulation after papaverine mentioned earlier in this report suggests that in this chamber, at least, the refractory period is prolonged. The development of block in the A-V junction tissue and within the ventricles might also be attributed, at least in part, to a prolongation of the refractory period in these regions. In fact the appearance of ectopic rhythms which we found may be due to a similar mechanism associated with reentry. For these reasons, a systematic analysis of the changes in the refractory period of the ventricles was undertaken.

For this purpose anesthetized dogs weighing 6½ to 15 kilo were prepared as in the preceding experiments. Similar care was used to keep the heart warm and moist. A Lewis interrupter was again employed to drive the auricles at a fixed rate throughout the experiment, about 50 beats faster than the sinus rate, so that the influence of heart rate which alters the refractory period could be excluded. The ventricles were stimulated with platinum hook electrodes inserted in the right ventricle or the apex of the left ventricle. The stimulating electrodes were connected in this group of experiments to a condenser and B battery attached to a metronome set at a constant rate. The rate used was a trifle over or under ½ of the rate of stimulation of the auricles by the Lewis interrupter. In this way the ventricular stimulation was out of phase with the auricular stimulation. The current strength was adjusted, by varying the capacity of the condenser so that the stimulus was definitely above the threshold strength necessary to give a response in late diastole, and this current strength was maintained constant throughout the experiment. The time of stimulation of the ventricle was recorded by inserting the primary of a Harvard inductorium in the stimulating circuit and connecting the secondary (at an appropriate distance from the primary) in series with one of the lead wires of the electrocardiograph.

The measurement of the refractory period was made by determining the interval between each ventricular stimulus and the onset of QRS of the preceding supraventricular beat and noting whether the stimulus was effective or not. For this purpose the results of a 3 minute

continuous observation immediately after the drug was given were compared with a like period before the administration. The heart cycle, which was fixed, was subdivided into intervals of 0.02 second. Each stimulus was placed in its proper time interval together with one notation whenever it was not effective and another when it led to an ectopic response.

In these measurements the interval from the beginning of ventricular electrical systole to the stimulus, the *Q-S* interval, is measured and not the interval to the response, the *Q-R* interval. Consequently the "latent period" between stimulus and measurable response, the *S-R* interval, is excluded. This arrangement of measurement is necessary in order to account for those stimuli not leading to responses. In order to correct this omission of the *S-R* interval, we have added 0.03 sec. to the *Q-S* interval and assumed this to be the *Q-R* interval. The graphs shown (figs. 2 to 6) are all corrected in this way. Actual measurements of a number of the 3 minute records demonstrated that the *S-R* interval was actually 0.03 ± 0.01 sec. regardless of whether the record was taken before or after the drug adminis-

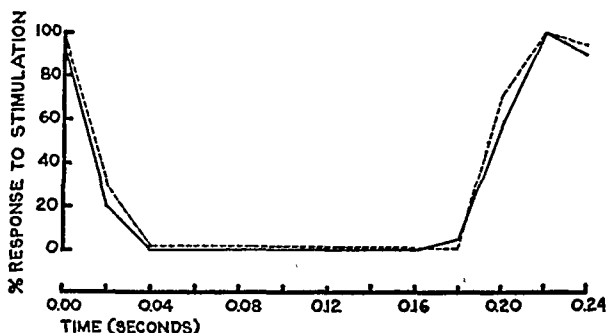


FIG. 2. THE EFFECT OF 5 CC. OF HYDROCHLORIC ACID (pH = 3.2) INTRAVENOUSLY ON THE REFRACTORY PERIOD OF THE VENTRICLES

Abscissae represent time, in seconds, in the cardiac cycle when the response occurred or was expected. (This was obtained by adding the "latent period" to the time of application of the stimulus.) Zero time is the onset of *QRS* of the supraventricular beats. The ordinates represent the percentage of responses obtained from the stimuli at these time intervals. The control graph is shown by the broken line and the effect after the drug by the solid line. The heart rate was maintained constant (250/min.) by stimulating the auricles artificially. The cycle length was 0.24 sec. throughout.

tration, and in the latter case irrespective of the dose of drug given. Nevertheless, it is possible that the time when response might have occurred but did not in our experiments, could have been longer than the value we used. Such an error, however, would not militate against the value of the charts showing alterations in the refractory phase. In constructing the graphs the percentage of responses were plotted for each *Q-R* interval of the heart cycle (0.02 seconds apart) and comparisons made between the graph after the injection of the drug and that of the control.

Two control experiments were made using 5 cc. of hydrochloric acid (pH = 3.2), the results of one of which is shown in figure 2. In neither experiment was any significant change in the refractory period observed.

A total of 14 experiments were made on the innervated heart, 2 with 27 mgm., 6 with 92 mgm., 5 with 157 mgm. and 1 with 260 mgm. Even in the experiments with 27 mgm. there was a tendency for the refractory period to lengthen slightly (fig. 3), and the effect became more noticeable with the larger doses (figs. 4 to 6)

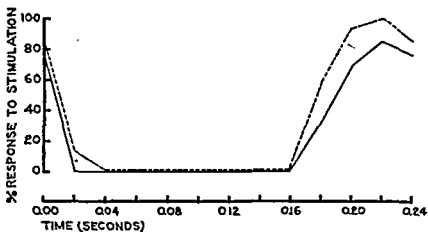


FIG. 3. THE EFFECT OF PAPAVERINE HYDROCHLORIDE (27 MG.M.) INTRAVENOUSLY ON THE REFRACTORY PERIOD OF THE VENTRICLES

Heart rate = 250/min., electrical cycle = 0.24 sec. Conventions as in figure 2.

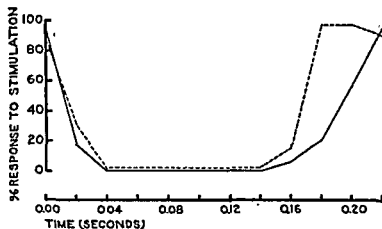


FIG. 4. EFFECT OF PAPAVERINE HYDROCHLORIDE (92 MG.M.) INTRAVENOUSLY ON REFRACTORY PERIOD OF VENTRICLES

Heart rate = 272/min., electrical cycle = 0.22 sec. Conventions as in figure 2.

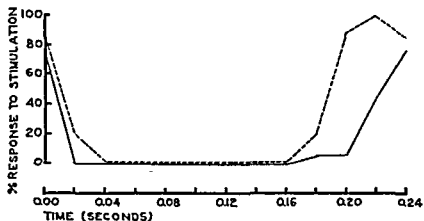


FIG. 5. EFFECT OF PAPAVERINE HYDROCHLORIDE (157 MG.M.) INTRAVENOUSLY ON THE REFRACTORY PERIOD OF VENTRICLES

Heart rate = 250/min., electrical cycle = 0.24 sec. Convention as in figure 2.

although in one of 6 experiments with 92 mgm. of the drug no measurable effect was seen.

In these figures it will be noted that some responses occur after electrical systole has begun (for 0.02 sec.). This may be due to errors in measurement but in all likelihood it is attributable to the fact that the ventricles have not been stimulated at that time in their entirety. Thus the extrinsic stimulus finds part of the ventricles still responsive and therefore can elicit an ectopic response. The conditions of spread of the auricular and ectopic impulses are sufficiently variable to account for the absence of response to the extrinsic stimulation on some occasions in this period of the heart cycle. This general interpretation is verified by the occurrence of fusion beats in this period.

That this effect is due to a direct action on the heart was demonstrated in the "physiological heart-lung preparation" described in section 1. In one experiment in which papaverine hydrochloride (27 mgm.) was given intravenously, a

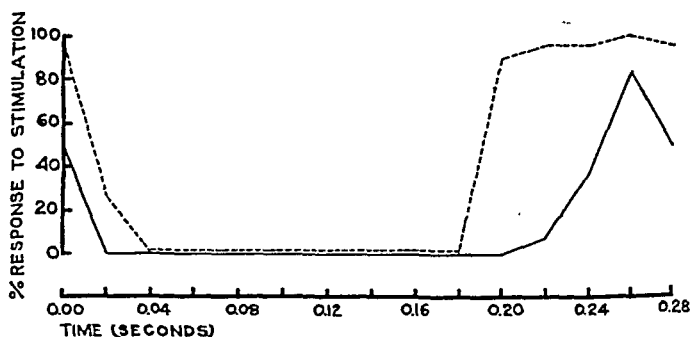


FIG. 6. EFFECT OF PAPAVERINE HYDROCHLORIDE (260 MGm.) INTRAVENOUSLY ON THE REFRACTORY PERIOD OF VENTRICLES

Heart rate = 214/min., electrical cycle = 0.28 sec. Convention as in figure 2.

definite lengthening of the refractory period was obtained in the first 3 minutes after the administration of the drug. The procedure for measuring the refractory period was the same as in the experiments in the intact animal. There can therefore be no doubt that the action of papaverine in prolonging the refractory phase of the ventricles is due to a direct action on the heart.

GENERAL DISCUSSION. The doses of papaverine used in these experiments are larger than those recommended for man in the New and Nonofficial Remedies (30 to 80 mgm. orally or hypodermically). However, larger doses have been utilized abroad; for example, Dopffel and Kutschera-Aichbergen (14) have given as much as 325 mgm. of eupaverine intravenously in clinical cases (eupaverine is at least as effective as papaverine), and we ourselves have used dosage of papaverine up to 227 mgm. in divided doses ten minutes apart in similar (unpublished) studies on man.

On the basis of our present and previous experiments papaverine hydrochloride has the following effects on the cardinal properties of heart muscle:

(1) It depresses A-V conductivity and intraventricular conductivity, which result in A-V and intraventricular block with larger doses. (2) It diminishes or abrogates ventricular ectopic beats induced either at a fixed part or different parts of the cardiac cycle. (3) It makes it more difficult to produce or maintain auricular fibrillation by means of a faradic current. (4) It restores regular beating of the ventricles after longstanding faradically produced ventricular fibrillation (provided cardiac nourishment is maintained). (5) It raises the threshold (measured by the distance of the secondary from the primary coil of the Harvard inductorium) for inducing ventricular fibrillation. (6) In its presence vigorous manual massage of the fibrillating ventricles will restore orderly synergic beating. (7) It may by itself, in toxic or subtoxic doses, lead to active ectopic ventricular rhythms (premature systoles, paroxysmal tachycardia and flutter and fibrillation). (8) In toxic doses, it can lead to cardiac standstill or complete A-V block. (9) Its toxic action occurs more readily in hearts that are ischemic. (10) These effects are primarily direct actions on the heart.

Papaverine hydrochloride thus appears to act as a myocardial depressant and, like most depressants, it can at the same time often cause ectopic rhythms. It appears to retard the recovery phase of the heart muscle and thereby lengthens the refractory phase of the ventricles and auricles. In addition, we have shown previously that it exerts a direct powerful and lasting dilating action on the coronary vessels (7).

From our present studies it would appear that several circumstances combine to make it more difficult to produce ventricular fibrillation after papaverine:

(1) The drug renders the heart less responsive to ectopic stimuli. This is extremely important if it is assumed that the effect is the same on ectopic stimuli generated within the heart as upon induced electrical stimuli; although not demonstrated, this seems to be highly probable. On a purely statistical basis, the chances of fibrillation will be reduced when the number of ventricular premature beats is diminished. The unresponsiveness may be the result of a focal block around the ectopic pacemaker preventing its impulse from spreading. The development of conduction with decrement from the ectopic focus may be invoked but this is not proven nor necessary. Of course, the ectopic focus may become depressed so that it discharges at a slower rate or becomes entirely inactive.

(2) It depresses conductivity in the ventricle. This is of course a two-edged weapon. It may enhance "exit block" and so render parasystolic rhythms ineffective (11) and thereby reduce the number of ectopic beats and on this account the chance of fibrillation. But, on the other hand, this in a heart already depressed may lead to the development of unidirectional block in the ventricle which is the background of reentry (11) and so may aggravate the frequency of premature beats and the chance of fibrillation. This is perhaps the way in which toxic doses of the drug operate and this may be the untoward effect which may result in diseased hearts.

(3) It lengthens the refractory period. This also is a two-edged weapon. On the one hand, by lengthening the refractory period it may abolish the circum-

stances of unidirectional block so essential for reentry, an important probable cause for ectopic rhythms and ventricular fibrillation. On the other hand, the lengthening of the refractory phase may, when its effects are unequal in neighboring units of the heart, actually lead to the development of unidirectional block (11) and so initiate ventricular fibrillation.

These several aspects of papaverine action may be separate phenomena or more likely are interrelated and different aspects of the same fundamental process. Nevertheless, it is possible to explain the beneficial effects of papaverine as a prophylactic and curative factor in ectopic rhythms and ventricular fibrillation on an analytic basis such as has been attempted. This analysis also serves as the background to account for the occurrence of ectopic rhythms seen with larger doses. The relation of these findings to clinical use must for obvious reasons be determined by clinical trial in diseased hearts. Only then can the indications and contraindications be properly worked out.

We have no direct observations upon the relation of papaverine to the vulnerable period of the heart which Wiggers and his associates (12) have demonstrated to be an important mechanism in the induction of fibrillation of the ventricles. Their theory that spontaneous ventricular fibrillation is precipitated because an ectopic stimulus, or even a normal impulse under abnormal conditions, comes in the vulnerable period, appears logical and supported by their evidence. If the action of the drug is to render the ventricles unresponsive for a longer period of the heart cycle as our results indicate, it would appear that the threshold of the vulnerable period would also be raised.

It is necessary further to point out that the coronary dilating action of the drug, by improving the nourishment of ischemic regions, might elevate the threshold of vulnerability and would also reduce the frequency of ectopic beats. On this account, it would appear that myocardial infarction and chronic coronary insufficiency would be especially favorable to the exhibition of papaverine, and McEachern and his associates (13) found such a benefit in dogs with coronary occlusion. This requires exploration clinically.

A word of caution should be interjected. Our experiences show that while papaverine tends to abolish ectopic rhythms and fibrillation, the drug when used in larger doses may lead to their occurrence. In this regard it is no different from other cardiac drugs, viz., digitalis, quinidine, etc.

SUMMARY

1. Papaverine depresses or annuls the responsiveness of the auricles to faradic stimulation.

2. In small doses it may augment A-V conductivity, but in larger doses it depresses A-V conductivity leading to partial or complete A-V block. Larger doses also cause intraventricular block.

3. It diminishes the irritability of the auricles and ventricles to extrinsic stimuli and prolongs the refractory period of the ventricles and auricles.

4. In the anesthetized dog breathing naturally and with natural beating of the heart, small doses of papaverine usually cause temporary sinus tachycardia.

Larger doses lead to sinus slowing or standstill sometimes with nodal escapes or nodal rhythm, active ectopic ventricular rhythms and even ventricular flutter and fibrillation. Death with toxic doses is due to cardiac or ventricular standstill or to terminal ventricular fibrillation of a peculiar type.

5. The favorable action of this drug in reversing artificially induced ventricular fibrillation, and in diminishing or abrogating ventricular premature contractions electrically induced, is explained as due to a depression of conductivity and irritability and to a prolongation of the refractory period of the ventricles.

6. Evidence is presented to show that these are primarily direct effects on the heart.

7. The possible prophylactic and therapeutic clinical application of papaverine hydrochloride in the management of ectopic rhythms and ventricular fibrillation is suggested, but caution is advised inasmuch as this drug may cause the very arrhythmias for which it may be recommended.

We are indebted to Dr. K. Jochim and Mr. M. Feinstein for their assistance.

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STUDIES ON VERATRUM ALKALOIDS

I. THE ACTION OF VERATRINE UPON THE ISOLATED MAMMALIAN HEART¹

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In the course of some experiments on the vagal action of veratrine it was noticed that veratrine had an effect upon the isolated mammalian heart which appeared similar to that of ouabain and other cardiac glycosides. This observation seemed of interest because the chemical configuration of the veratrum alkaloids is quite different from that of the cardiac glycosides. A search of the literature revealed that the cardiac action of veratrine described in this paper has not been thoroughly investigated, although experiments of several authors have a bearing on it.

A decrease in heart rate and an increase in the amplitude of the ventricular contraction was noticed by Hedbom (1) and by Kuliabko (2) when veratrinum hydrochloridum Merck was given to the Langendorff preparation of the heart of rabbits and cats. Ishihara and Pick (3), Wachstein (4), and Goldenberg and Rothberger (5) studied the effect of veratrine on Purkinje fibers. Wachstein suspended in Soejima solution sections of the A-V bundle of dogs and recorded the spontaneous contractions. As a rule veratrine sulfate caused a decrease in the rate of contraction, but occasionally a slight and transient increase was noted. The lowest effective concentrations considerably increased the amplitude of the contractions. Gibert-Queraltó and Pescador (6) made a few experiments with the heart-lung preparation of the dog, and in the suspension curves of the right ventricle they found a marked increase in the amplitude of the ventricular contraction when veratrine sulfate was administered. They also noticed a decrease in heart rate and an increase in coronary flow.

One of the reasons why the action of veratrine described here escaped closer attention is that the strong vagal action of the drug on the intact circulatory system of the mammal entirely obscures the direct cardiac action (7). Furthermore, as with the cardiac glycosides, this effect is not marked unless the heart shows signs of failure.

Twenty-two experiments were carried out in order to establish some of the fundamental effects of non-toxic doses of veratrine upon the isolated and denervated dog heart in the form of the heart-lung preparation.

METHODS. Dogs between 6.5 kgm. and 13 kgm. were used. They were anesthetized with 0.09 gram of chloralose per kilogram of body weight. The heart-lung preparation was made as described by Patterson and Starling (8), defibrinated blood being used to feed the

¹ This work was done under the auspices of the University Committee on Pharmacotherapy.

heart. The total amount of blood in the system was between 450 and 1000 cc. Arterial pressure was recorded by a mercury manometer, pressure from the right auricle by a water manometer, and pressure from the left auricle by a bromoform manometer. A bromoform manometer was used to record pulmonary pressure from a side branch of the pulmonary artery.

The venous blood supply to the heart usually was maintained by gravity flow from an inflow vessel in which the level, hereafter referred to as "inflow level," was kept at a constant height above the heart. To determine the ability of the heart to respond to increased venous blood supply the inflow level was raised 50 mm. and 100 mm. above the basal level (for details see [9]). When it was necessary to maintain a constant venous blood supply throughout the experiment, the blood was pumped to the heart at a constant rate, following the procedure of E. A. Muller (10). The heart volume was recorded by a Henderson cardiometer attached to a small spirometer constructed so that a change of one millimeter in the position of the pointer corresponded to a change of 1 cc. in volume (for details see [10]). The systemic output of the heart (output of left ventricle minus coronary flow) was recorded by a Weese Stromuhr (11). In order to observe changes in coronary flow, a Morawitz cannula was introduced into the coronary sinus and the outflow measured by a Condon recorder. The amount of coronary blood flowing from the Morawitz cannula was taken to be 60 per cent of the total coronary flow (12). In our figures and tables the values for total coronary flow have been calculated on the basis of this assumption.

Failure of the heart was either spontaneous, occurring during the course of the experiment, or induced by adding 0.05 gram to 0.2 gram of sodium pentobarbital (nembutal) to the blood in the heart-lung system [for technical details concerning this failure see (9), (13), (14)].

Most of our experiments were made with veratrine hydrochloride* obtained from the seeds of *Schoenocaulon officinale* Gray. This veratrine is said to be a mixture of the following alkaloids: cevadine, veratridine, cevadilline, sabadine, and cevine.² Identical results were obtained in a few experiments with an old sample of veratrine sulfate.³ Unless otherwise indicated, the veratrine was injected into the blood before it entered the venous reservoir.

I. ACTION UPON OUTPUT OF THE HEART AND UPON CORONARY BLOOD FLOW.
If no signs of heart failure are noticeable in the heart-lung preparation, even a substance which improves the activity of the heart muscle cannot bring about a marked increase in output. Under these conditions the response to an increase in venous blood supply often indicates more clearly that a drug has improved the heart. Thus in figure 1 a dose of 0.3 mgm. of veratrine sulfate increased the systemic output only from 555 to 600 cc. per minute, but when the venous blood supply was increased by steps, there was a distinctly smaller rise in auricular pressure with each step than was observed previous to the administration of veratrine. The effect of veratrine upon total output in a normal heart is illustrated by figure 2. In this experiment a dose of 0.2 mgm. of veratrine hydrochloride increased the coronary flow slightly. There was no significant change in systemic output, rate, mean arterial pressure, or pulmonary pressure. While the total output, calculated from the systemic output and the coronary flow, showed no measurable increase, there was a distinct decrease in the right auricular pressure.

* The Merck Index, Fifth Edition. 1940, p. 574. We are indebted to Merck and Co., Rahway, New Jersey, for furnishing us with the veratrine hydrochloride.

³ This veratrine sulfate was manufactured by E. Merck, Darmstadt, Germany, its composition is unknown to us.

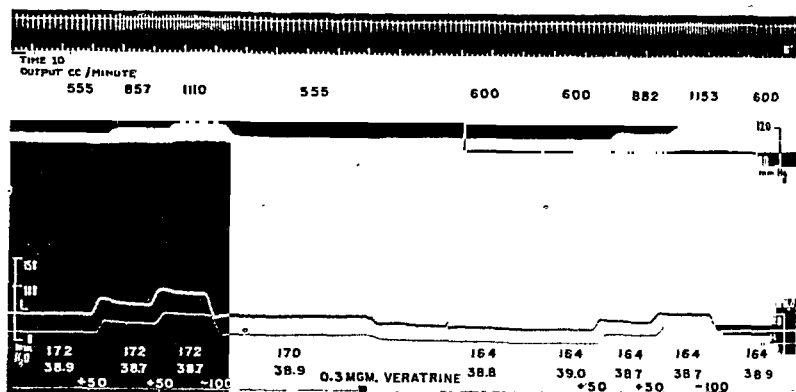


FIG. 1. EXPERIMENT 2

Action of veratrine on the normal heart. Heart-lung preparation. Weight of heart-lung dog 10.0 kgm. Approximate blood volume 700 cc. Tracings from top to bottom: systemic output, each signal indicating 100 cc.; time in 10-second intervals; arterial blood pressure (scale on right in mm. of mercury); left auricular pressure L (scale on left in mm. of water); right auricular pressure R (scale on right in mm. of water). The horizontal rows of figures, from top to bottom, indicate systemic output in cc. per minute, heart rate per minute, temperature of the blood in centigrade, and changes in inflow level in mm. At signal 0.3 mgm. of veratrine sulfate was injected.

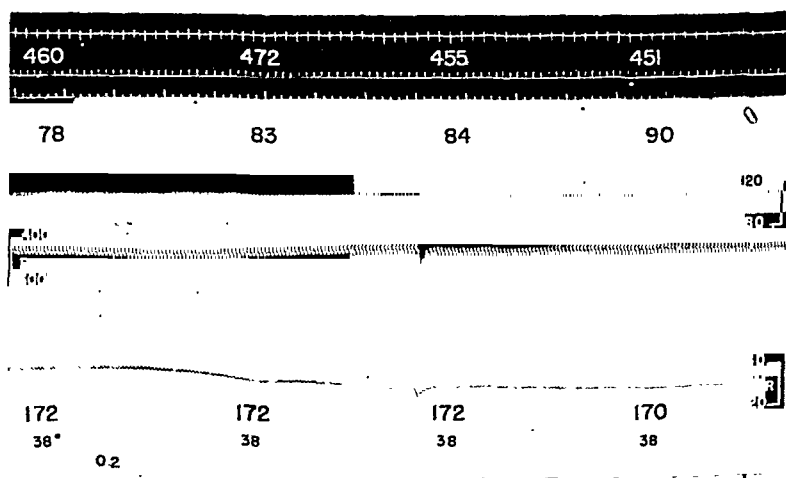


FIG. 2 EXPERIMENT 20

Action of veratrine on the normal heart. Heart-lung preparation. Weight of heart-lung dog 9.5 kgm. Approximate blood volume 800 cc. Arterial resistance 82 mm. of mercury. Tracings from top to bottom: systemic output, each signal indicating 100 cc.; coronary flow, each signal indicating 8.0 cc.; time in 10-second intervals; arterial blood pressure (scale on right in mm. of mercury); pulmonary arterial pressure P (scale on left in mm. of water); right auricular pressure R (scale on right in mm. of water). Horizontal rows of figures from top to bottom: systemic output in cc. per minute, coronary flow in cc. per minute, heart rate per minute, temperature in centigrade. At first signal 0.2 mgm. of veratrine hydrochloride was injected. At second signal the drum was stopped for 5 minutes.

Although the changes in auricular pressure and in output were slight in the normal heart, they were indicative of the capacity of veratrine to relieve heart failure. This is evident in the experiment of figure 3, in which spontaneous failure was allowed to develop. The total output fell within one and one-half hours to 56 per cent of the original value. The first dose of 0.3 mgm. of veratrine hydrochloride caused an increase up to 79 per cent of the original output, and a subsequent dose of 0.3 mgm. restored the original total output. While the heart rate decreased 10 per cent the stroke volume rose 90 per cent, exceeding the stroke volume of the normal heart. At the same time the size of the heart diminished.

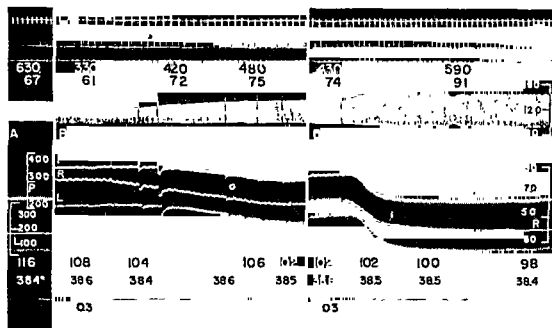


FIG 3. EXPERIMENT 22

We made a more extensive study of the effect of veratrine upon heart failure caused by derivatives of barbituric acid. In nine experiments with varying degrees of failure the effect of veratrine was consistent. The first part of table 1, which is a record of a representative experiment, shows the well-known effects of a barbituric acid compound upon systemic output, auricular pressure, and diastolic volume of the heart. As the dilatation of the heart progressed, its response to the increase in venous blood supply deteriorated. At the basal inflow level immediately before the administration of 0.2 mgm. of veratrine hydrochloride the systemic output was only 80 per cent of the original value. Within two minutes after veratrine was given the systemic output rose to 96

per cent of the normal output, while simultaneously the auricular pressure decreased and the diastolic volume of the ventricles diminished. The therapeutic effect upon the activity of the heart is clearly shown in the marked improvement of the response to the increase in venous blood supply.

The action of veratrine upon total output in nembutal failure is apparent from table 3. In this experiment 0.1 gram of nembutal caused a decrease to 68 per cent of the original output. The first dose of 0.3 mgm. of veratrine hydrochloride increased the output within 3 minutes, to 109 per cent. Forty-five

TABLE 1

Experiment 11. Action of veratrine on the heart in nembutal failure

Heart-lung preparation. Weight of the heart-lung dog 9.2 kgm. Weight of the heart 76 grams. Arterial resistance 80 mm. of mercury. Approximate blood volume 600 cc.

TIME	TEMPERATURE	HEART RATE	INCREASE IN INFLOW LEVEL	SYSTEMIC OUTPUT	ARTERIAL PRESSURE	RIGHT AURICULAR PRESSURE	LEFT AURICULAR PRESSURE	INCREASE IN DIASTOLIC HEART VOLUME
min.	°C.	per min.	mm.	cc./min.	mm. Hg	mm. H ₂ O	mm. H ₂ O	cc.
0	38.6	154	0	428	108	52	45	0
2	38.8	154	50	600	110	65	65	3
5	38.8	156	0	420	107	52	45	0
6		Nembutal 50 mgm.						
11	38.8	160	0	375	104	98	120	23
13	38.8	158	0	368	103	99	122	24
15	38.8	156	50	508	107	142	175	28
17	39.0	156	100	571	108	218	205	33
21	38.8	164	0	363	103	104	130	26
22	39.0	162	50	454	106	162	192	31
24	39.0	164	100	517	108	244	205	34
27	36.7	162	0	340	101	113	135	26
29	38.5	158	0	337	100	116	135	27
30		Veratrine hydrochloride 0.2 mgm.						
32	38.7	152	0	413	104	63	85	13
34		148	0	392	103	63	80	14
37	38.6	148	0	377	102	64	75	14
38	38.2	146	50	535	106	82	100	18
40	39.0	144	100	681	110	113	125	23
44	38.7	144	0	363	102	75	85	18

minutes later, when the heart again showed signs of failure, the second dose of 0.3 mgm. caused a rise from 61 per cent to 109 per cent of the normal total output.

In one experiment (see table 2) it was possible to observe the action of veratrine upon the systemic output of a heart with symptoms of failure accompanied by auricular fibrillation of unknown cause. Two doses of 0.1 mgm. each of veratrine sulfate given in succession increased the systemic output to 250 per cent of the original value. In spite of the persisting auricular fibrillation the ventricular rate became more regular and there was a marked improvement in the response of the heart to the increase in venous blood supply.

Coronary blood flow was measured in 6 experiments. The 4 experiments of

figure 4 contain the essential results. In the normal heart non-toxic doses of veratrine had no marked effect upon coronary flow. If an effect was noticeable, it consisted of a slight and transient increase. In the failing heart (nembutal) a therapeutic dose of veratrine augmented the outflow from the Morawitz cannula. This effect was transient and coincided with the decrease in heart volume. Maximal values of outflow were reached within 15 minutes. Repeated administration of the same dose showed diminution of the intensity and duration

TABLE 2

Experiment 8. Action of veratrine and quinidine in auricular fibrillation

Heart-lung preparation Dog 13.0 kgm Blood volume approximately 450 cc. Arterial resistance 86 mm of mercury. Weight of the heart 86 grams.

TIME	VENTRICULAR RATE PER MINUTE*	INCREASE IN INFLOW LEVEL	SYSTEMIC OUTPUT	AURICULAR PRESSURE		MEAN ARTERIAL PRESSURE	TEMPERATURE
				Right	Left		
min.		mm	cc/min	mm H ₂ O	mm. H ₂ O	mm Hg	°C.
1		0	240			100	37.8
4		0	240			100	
5		Veratrine sulfate 0.1 mgm.					
8		0	331	82	120	110	37.8
11		0	400	72	92	115	37.8
16		0	487	63	68	116	37.8
18		50	770	71	80	121	37.8
20		100	980	75	80	124	37.8
21	240	0	468	64	76	116	37.8
25		Veratrine sulfate 0.1 mgm.					
29	240	0	593	46	36	118	37.7
32	210	50	845	54	55	123	37.7
35	240	100	1090	60	53	126	37.8
39	240	0	600	48	40	118	38.0
40		Quinidine sulfate 10 mgm.					
45	200	0	612	47	36	118	37.8
46		Auricular fibrillation ceases					
49	120	0	612	43	40	118	37.6
51	120	50	895	46	46	124	37.5
53	120	100	1132	50	58	128	37.5
55	124	0	612	41	36	118	37.9

* During the first 19 minutes of the experiment the ventricular rate was very irregular and so frequent that it could not be counted

of the effect. The rise in mean arterial pressure which occurred simultaneously with the increase in coronary blood flow was too slight to account for more than a small part of the increase.

II. ACTION UPON RATE OF THE HEART. Effective but non-toxic initial doses of veratrine (see table 4) had no uniform influence upon heart rate in the 13 experiments performed on normal hearts. In 8 experiments the rate decreased, in 3 it did not change, and in 2 it increased. As the initial dose approached the toxic dose the heart responded more readily with an increase in rate. Doses

subsequent to the initial dose usually further decreased the rate, but in some experiments the rate was not altered, and in others it increased.

In nembutal failure the initial effective but non-toxic dose of veratrine caused a decrease in heart rate in 7 out of 9 experiments; in the other 2 there was no change in rate. Subsequent doses tended to decrease the heart rate to lower levels than in the normal heart. In the experiment of table 3 the rate dropped from 148 per minute to a regular sinus rate of 52 per minute, after a total amount of 3.5 mgm. of veratrine hydrochloride had been given.

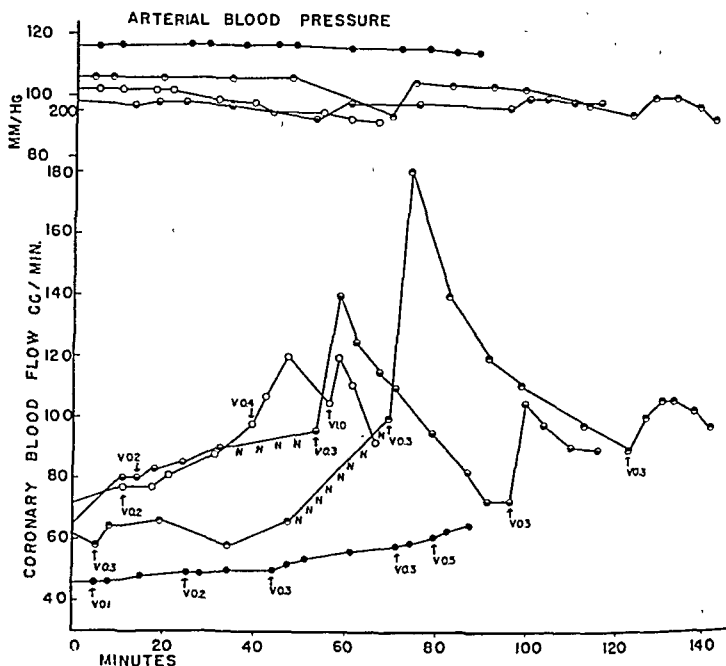


FIG. 4. ACTION OF VERATRINE ON CORONARY FLOW

Heart-lung preparation. The arrows indicate when veratrine was given. Each N indicates injection of 25 mgm. of nembutal. The figures after V indicate in mgm. the dose of veratrine sulfate in Experiment 4 (●), the dose of veratrine hydrochloride in Experiment 5 (○), Experiment 20 (◐), and Experiment 21 (◑).

In the experiment of table 2 two doses of 0.1 mgm. each of veratrine sulfate given in succession did not stop auricular fibrillation. While the ventricular rate became more regular, it did not decrease below 240 per minute.

III. ACTION UPON PULMONARY CIRCULATION. In the normal heart-lung preparation no marked changes in pulmonary arterial pressure followed the administration of non-toxic or even slightly toxic doses of veratrine (see figs. 2 and 7). In several experiments a decrease in pressure of a few millimeters of water was noticed, particularly if the venous blood supply was kept constant, as in the experiment of figure 6.

The development of heart failure in the heart-lung preparation was usually accompanied by a rise in pulmonary arterial pressure. Under this condition an adequate dose of veratrine restored normal pressure. The drop in pulmonary arterial pressure was sometimes preceded by a slight rise, which lasted for about one minute and coincided with the beginning of the increase in the total output of the heart. As the effect of the drug wore off, the pulmonary pressure again rose. Subsequent doses of veratrine were less effective in intensity as well as in duration. The magnitude of the changes observed can best be judged from the experiments of figure 3 and table 3. These experiments prove that veratrine reduced the resistance in the pulmonary circulation.

The magnitude of the change in output is not dependent upon the degree of the hemodynamic changes in the pulmonary circulation. This is evident if the action of the first dose of 0.3 mgm. of veratrine hydrochloride in the experiment of table 3 is compared with the effect of the second dose of 0.3 mgm. As a result of the first dose the total output increased 60 per cent, while the pulmonary pressure decreased 21 per cent; as a result of the second dose the total output increased 77 per cent, reaching the same absolute value as after the first dose, while the pulmonary arterial pressure, which had returned to the previous level, dropped only 5 per cent.

IV. ACTION UPON DIASTOLIC VOLUME AND UPON WORK OF THE HEART. One of the characteristic effects of a non-toxic dose of veratrine is a decrease in the diastolic volume of the heart. Figure 5 illustrates this effect in an experiment in which the heart showed no apparent signs of failure and changes in systemic output, rate, and pulmonary arterial pressure were either absent or insignificant. This action of veratrine is much more pronounced in the failing heart, with its marked dilatation (see table 1). However, in heart failure the interpretation of the change in diastolic volume is complicated by the action of veratrine upon output, heart rate, and pulmonary arterial pressure, the last having a particularly strong influence upon the size of the right ventricle (15).

If the blood enters the heart by gravity flow, alterations in heart rate or in stroke volume lead to changes in the venous blood supply. For an accurate evaluation of the influence of a substance upon the diastolic volume of the heart it is necessary to keep the venous blood supply constant. In the experiment of figure 6 this was done by pumping the blood into the right atrium at a constant rate. The systemic output remained at 610 cc. throughout the experimental period. Three-tenths mgm. of veratrine hydrochloride caused a maximal decrease in diastolic heart volume of 17 cc., which was reached within 6 minutes. Immediately after the injection the pulmonary arterial pressure decreased slightly but had already returned to its original level when the heart volume was smallest. Neither the change in pulmonary arterial pressure nor the slight increase in rate, indicating a decrease in stroke volume, can have significantly modified the reaction.

From these observations it must be concluded that veratrine has a direct action upon the ventricular muscle of the mammalian heart, leading to a decrease in the diastolic ventricular volume.

The work of the heart was calculated from the total output and from the aortic pressure and pulmonary arterial pressure. The energy expended in imparting a certain velocity to the blood was not considered. Consistent results were obtained in four experiments, one of which is illustrated by table 3.

In the normal heart 0.2 mgm. of veratrine hydrochloride did not significantly increase the work of the heart. Nembutal markedly reduced it. Three doses of 0.3 mgm. of veratrine hydrochloride given in succession all uniformly increased

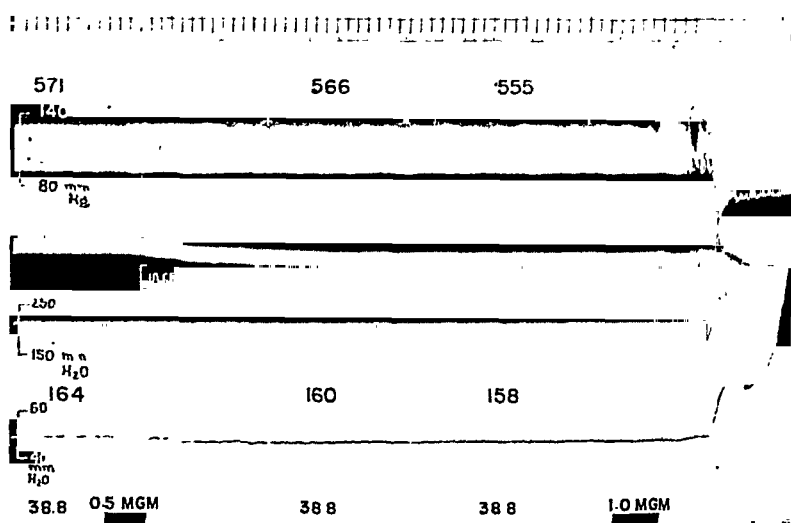


FIG. 5. EXPERIMENT 12

Action of a therapeutic dose of veratrine upon heart volume and toxic action resulting in ventricular fibrillation. Heart-lung preparation. Weight of heart-lung dog 6.9 kgm. Weight of the heart 64 grams. Approximate blood volume 750 cc. Arterial resistance 90 mm. of mercury. Tracings from top to bottom: systemic output, each signal indicating 100 cc.; time in 10-second intervals; arterial blood pressure (scale on left in mm. of mercury); heart volume (scale under tracing in cc.); pulmonary arterial pressure (scale on left in mm. of water); right auricular pressure (scale on left in mm. of water). The horizontal rows of figures indicate, from top to bottom: systemic output in cc. per minute, heart rate per minute, temperature of the blood in centigrade. At signals 0.5 and 1.0 mgm. of veratrine hydrochloride was injected. Two doses of 0.25 mgm. each of veratrine hydrochloride were given before those indicated in the tracing.

the work. Maximal effects were reached within 3 minutes. As the heart rate in this experiment decreased under the influence of veratrine, the stroke volume increased accordingly. However, if short intervals are considered, such as the time between the administration of veratrine and the maximal effect, the change in rate could not have accounted for more than a small part of the increase in stroke volume.

V. DOSAGE AND DURATION OF ACTION. 1. *Initial effective and therapeutic dose.* In table 4 are recorded the initial effective but non-toxic doses of veratrine

used in our experiments. This dosage range also represents the initial effective or "therapeutic" dose for the failing heart. One-tenth mgm. of veratrine sulfate or veratrine hydrochloride was the smallest effective dose observed, while the smallest toxic dose was found to be about 0.5 mgm. of veratrine hydrochloride (1.0 mgm. per liter of blood, or 0.75 mgm. per 100 grams of heart muscle).

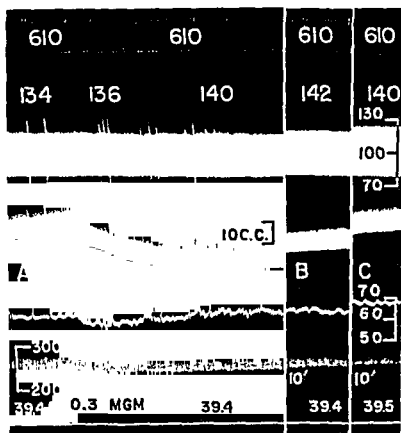


FIG. 6 EXPERIMENT 16

arterial pressure (scale on right in mm. of mercury); heart volume (scale above the tracing minutes after B

Transient periodic irregularities and increase in heart rate (see fig. 7) were the characteristic effects of the smallest toxic dose. The appearance of the toxic effect was found to be dependent upon the concentration of veratrine first reaching the heart, rather than upon the total dose. In contrast to the usual procedure the injection in the experiment of figure 7 was made into the blood stream shortly before it entered the right atrium. The concentration of veratrine hydrochloride reaching the coronary arteries the first time was about 1:350,000. If the veratrine had been uniformly distributed, the total dose of

0.3 mgm. (0.43 mgm. per liter of blood, or 0.31 mgm. per 100 grams of heart) would have been well within the non-toxic range.

TABLE 3

Experiment 20. Action of veratrine hydrochloride on the normal heart and on the heart in nembital failure

TIME	SYSTEMIC OUTPUT	CORO-NARY FLOW	TOTAL OUTPUT	HEART RATE	STROKE VOLUME	MEAN ARTERIAL PRESSURE	PULMO-NARY ARTERIAL PRESSURE	RIGHT AURICULAR PRESSURE	WORK OF THE HEART IN KILOGRAM-METER PER MINUTE		
									Total	Left heart	Right heart
min.	cc./min.	cc./min.	cc./min.	per min.	cc.	mm. Hg	mm. H ₂ O	mm. H ₂ O			
0	487	63	550	180	3.1	98	391	37	0.95	0.73	0.22
11	468	78	546	172	3.2	97	366	39			
13	460	78	538	172	3.1	97	362	40	0.91	0.71	0.20
13	Veratrine hydrochloride 0.2 mgm. (0.26 mgm./liter of blood; approx. blood vol. = 750 cc.)										
16	572	83	555	172	3.2	97	364	33	0.93	0.73	0.20
18	458	83	541	172	3.1	98	371	30			
24	451	85	536	170	3.2	98	371	30			
32	451	90	541	170	3.2	98	371	34	0.93	0.72	0.21
33	Nembital 25 mgm.										
36	Nembital 25 mgm.										
40	Nembital 25 mgm.										
43	Nembital 25 mgm.										
45	270	96	366	148	2.5	95	469	144	0.66	0.49	0.17
46	Veratrine hydrochloride 0.3 mgm. (0.42 mgm./liter of blood; approx. blood vol. = 700 cc.)										
47	350	120	470	144	3.3	97	490	120	0.85	0.62	0.23
49	451	140	590	140	4.2	98	369	64	1.01	0.79	0.22
52	451	130	581	138	4.2	98	378	69			
58	451	116	567	136	4.2	98	385	79			
62	451	110	561	134	4.2	98	406	84	0.97	0.75	0.22
69	443	95	538	134	4.0	98	420	96			
75	420	88	508	132	3.9	98	437	107			
87	345	83	428	132	3.2	98	465	133	0.77	0.57	0.20
91	324	73	397	134	2.9	98	458	140			
93	290	73	363	134	2.7	97	465	143			
95	258	73	331	134	2.5	97	471	150	0.60	0.44	0.16
96	Veratrine hydrochloride 0.3 mgm. (0.54 mgm./liter of blood; approx. blood vol. = 550 cc.)										
99	483	105	588	124	4.7	100	448	89	1.06	0.80	0.26
103	428	98	526	116	4.5	100	459	102			
109	351	91	442	112	3.9	99	471	120			
115	252	90	342	108	3.4	99	464	151	0.62	0.46	0.16
116	Veratrine hydrochloride 0.3 mgm. (0.6 mgm./liter of blood; approx. blood vol. = 500 cc.)										
119	408	96	504	92	5.5	100	459	117	0.91	0.68	0.23
122	397	91	488	88	5.5	100	448	111			

2. *Intensity and duration of action with repeated administration.* The action of a fully effective dose of veratrine given initially lasted for 30 to 50 minutes; if the same dose was repeated, intensity and duration of effect usually diminished.

In the normal heart where veratrine has no influence upon coronary blood flow, the changes in heart volume are suitable for the estimation of the intensity and duration of action, provided the venous blood supply is kept constant. Thus in the experiment of figure 6 a dose of 0.3 mgm. of veratrine hydrochloride (0.46 mgm. per liter of blood) caused a decrease in diastolic heart volume of 17 cc., and the heart volume returned to the original value after 35 minutes. Fifty-four minutes after the first dose, 0.3 mgm. of veratrine hydrochloride (0.5 mgm. per liter of blood) caused a decrease in diastolic heart volume of only 10 cc., and the subsequent dilatation proceeded faster than after the first dose.

TABLE 4

Initial effective doses of veratrine hydrochloride producing no toxic effect

EXP. NO.	DOSE	DOSE PER LITER OF BLOOD	DOSE PER 100 GRAMS HEART
	mgm.	mgm.	mgm.
4*	0.1	0.11	0.080
16	0.1	0.13	0.086
17	0.1	0.15	0.16
3*	0.1	0.2	0.11†
6	0.1	0.2	0.2‡
7	0.2	0.23	
20	0.2	0.26	
10	0.2	0.28	0.13
19	0.2	0.28	0.20
8	0.2	0.28	0.26
5	0.2	0.33	0.15
11	0.2	0.33	0.26‡
12	0.25	0.33	0.30
1*	0.3	0.33	0.35‡
18	0.3	0.42	0.27‡
23	0.3	0.42	0.3‡
2*	0.3	0.43	
21	0.3	0.44	0.31‡
22	0.3	0.52	0.32**

* In this experiment veratrine sulfate was used

† Auricular fibrillation.

‡ Nembutal failure.

§ Histamine failure.

** Spontaneous failure.

In the failing heart changes in total output, coronary flow, pulmonary pressure, and auricular pressure can be used to investigate duration and intensity of action. In the experiment with spontaneous heart failure (fig. 3) the action of the fully effective second dose of 0.3 mgm. of veratrine hydrochloride (0.54 mgm. per liter of blood) lasted 40 minutes. The following dose of 0.3 mgm. (0.61 mgm. per liter of blood) had no significant effect; while 0.6 mgm. (1.2 mgm. per liter of blood) administered four minutes later was less active than the second dose, the effect lasting only 20 minutes. In the experiment of table 3 the action of the first therapeutic dose of 0.3 mgm. lasted about 45 minutes; the action of the second dose of 0.3 mgm., while equally effective in intensity, lasted 20 minutes.

The third dose of 0.3 mgm. was distinctly less active than the two preceding doses.

The decrease in rate caused by non-toxic or therapeutic doses did not seem to be entirely reversible. However, the interpretation is complicated in that the heart rate in the heart-lung preparation, in spite of the constancy of the temperature, does not stay at a constant level but gradually decreases. In

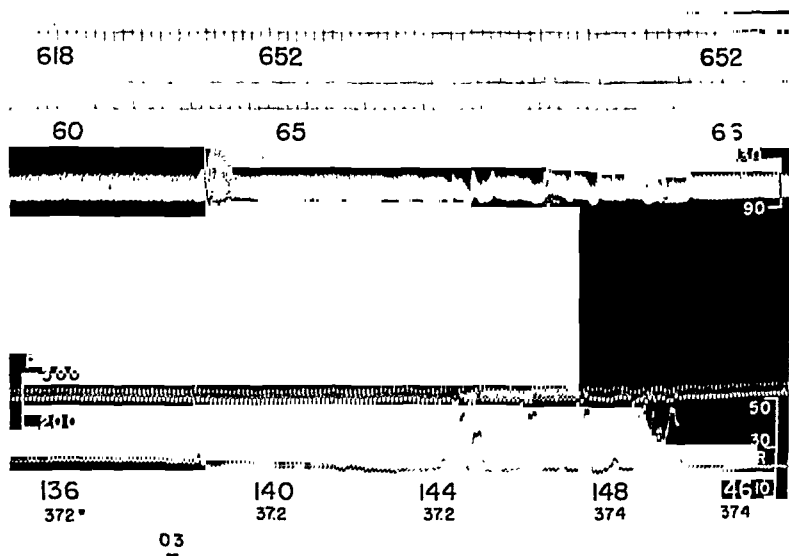


FIG. 7. EXPERIMENT 21

Toxic effect of veratrine characteristic of a minimal toxic dose. Heart-lung preparation. Weight of the heart-lung dog, 14.0 kgm. Weight of the heart, 96 grams. Approximate blood volume 700 cc. Arterial resistance 80 mm. of mercury. Tracings from top to bottom: systemic output, each signal indicating 100 cc.; coronary flow, each signal indicating 8.4 cc.; time in 10-second intervals. Arterial blood pressure (scale on left in mm. of mercury); pulmonary arterial pressure P (scale on right in mm. of water); right auricular pressure R (scale on right in mm. of water). The horizontal rows of figures indicate, from top to bottom: systemic output in cc. per minute, coronary flow in cc. per minute, heart rate per minute, and temperature in centigrade. At signal 0.3 mgm. of veratrine hydrochloride was injected into the tube carrying the venous blood supply from the reservoir to the heart.

cases where an increase in rate was caused by a toxic concentration of veratrine, as in fig. 7, this effect wore off within 30 minutes.

DISCUSSION. From the statements of Hedbom and of Kuliabko, it is impossible to ascertain what concentrations of veratrine hydrochloride they used. Ishihara and Pick applied 1:200,000 to 1:400,000 to the Purkinje fibers of dogs and rabbits; Goldenberg and Rothberger used 1:100,000 to 1:250,000, and Wachstein, 1:235,000 to 1:1 million veratrine sulfate on sections of the A-V conduction bundle of dogs. Gibert-Queraltó and Pescador gave repeated doses of 0.25 mgm. of veratrine sulfate in heart-lung preparations similar to our own

technic. They fail to mention, however, the total amount of blood in the heart-lung system.

From table 4 it is apparent that our effective concentrations of veratrine hydrochloride or veratrine sulfate ranged between 1:9 million and 1:2.5 million, while the toxic range began at a concentration of 1:1 million.

The most important effect of non-toxic or therapeutic doses of veratrine observed in our experiments is the effect upon the heart muscle leading to a decrease in the diastolic volume of the heart, which is in part due to a direct action of the alkaloid upon the muscle of the heart. The experiments on isolated parts of heart muscle, particularly those of Wachstein (4), substantiate this fact. As a result of this action the ventricular muscle can more readily adjust the output of the heart to the venous blood supply, thereby effecting in the failing heart a striking increase in total output and a marked improvement of the work.

This action of veratrine upon the heart muscle is similar to that of the cardiac glycosides. Like these, veratrine reaches its full effect on the muscular activity of the heart after a period of several minutes, during which the action gradually becomes more pronounced. In our experience an initial dose of 0.2 to 0.3 mgm. of veratrine hydrochloride approximately corresponds in the intensity of its therapeutic effect to that of 0.05 mgm. of ouabain.

The action on pulmonary arterial pressure which is in part attributable to a decrease in the resistance of the pulmonary circulation is similar to that observed by Gollwitzer-Meier and Krüger (13) with strophanthin under comparable experimental conditions.

The action of non-toxic doses of veratrine is more readily reversible than the action of corresponding doses of cardiac glycosides. Veratrine is unlike the cardiac glycosides in that the intensity and the duration of its action diminish with repeated non-toxic doses.

According to Gibert-Queraltó and Pescador veratrine increases coronary blood flow. We found this to be true of the failing heart, while in the normal heart not even slightly toxic doses caused a significant change.

SUMMARY

The cardiac action of non-toxic doses of veratrine was studied in the heart-lung preparation of the dog. The most important phase of this action is an effect upon the heart muscle which, particularly in the failing heart, increases the total output and improves the work of the heart, simultaneously with a decrease in the diastolic ventricular volume. The auricular pressure decreases. This action is similar to, although not identical with, the action of cardiac glycosides upon the mammalian heart.

The action of veratrine upon heart rate is not consistent; as a rule there is a decrease in rate.

The coronary flow in the normal heart remains unchanged, while in the failing heart veratrine causes a transient increase.

In the normal heart-lung preparation the pulmonary arterial resistance either remains unchanged or is slightly reduced. If a marked increase in pulmonary

pressure accompanies the development of heart failure, veratrine causes a marked reduction in the resistance of the pulmonary arteries.

The effective, therapeutic, and toxic doses are reported, and the intensity and duration of action with administration of single and repeated doses of veratrine are discussed.

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ACTION OF PHENYLCINCHONINIC ACID IN PREVENTING EXPERIMENTALLY PRODUCED CONVULSIONS IN RABBITS

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Bromides, phenobarbital and other anticonvulsants with the exception, among a few others, of sodium diphenyl hydantoinate, prevent experimental convulsions in animals by producing various degree of narcosis. In searching for another anticonvulsant that will not at the same time produce a narcotic effect, we have studied the effect of phenylcinchoninic acid in preventing convulsions produced by intravenous injection of metrazol and thujone into rabbits. Since the minimal dose of convulsant agents as thujone and metrazol is not constant because of inherent or acquired refractiveness, it has been suggested by Lennox, Nelson and Beetham (1) that statistical treatment rather than individual reports be used in the study of experimentally produced convulsions. Acquired refractiveness has been avoided in our studies by using only animals not previously exposed to any other experiment.

The observed attacks were classified as follows: Only a hunching backward as *no. 1*; twitching of ears and head as *no. 2*; myoclonic jerking of the muscles of the head, neck and extremities, the animal remaining erect as *no. 3*; a more severe myoclonic jerking of these parts, the animal remaining erect, as *no. 4*; and a complete clonic-tonic-clonic convulsion, the animal falling to the ground as *no. 5*. Each animal in which an anticonvulsant drug was injected previous to the injection of metrazol was controlled by an animal in which only the minimal convulsant dose of metrazol was injected.

From a study of 100 animals we established the minimal convulsant dose to be 13.2 mg. of metrazol given intravenously per kilo body weight of rabbit. There ensued 74 (74 per cent) complete convulsions (*no. 5*), severe myoclonic standing seizures (*no. 4*) in 9 (9 per cent), 13 (13 per cent) mild myoclonic standing seizures (*no. 3*), and 4 (4 per cent) twitchings (*no. 2*) (fig. 1).

Phenylcinchoninic acid (2-phenylquinoline-4 carboxylic acid), known as cinchophen or atophan, was first synthesized by Doebner and Gieseke (2). It has been widely used in the treatment of gout but because of its seemingly high toxicity its use has generally been discontinued. Following administration, it is excreted in the urine, in the form of 2 phenyl-8-hydroxy-4 quinoline carboxylic acid and hydroxy pyridinuric acid (3). In rabbits it is toxic in doses of 300-350 mg. per kilo. The minimal active dose is given as from 150-200 mg. per kilo and the lethal dose of from 500-600 mg. per kilo. When toxic doses are administered subcutaneously to a rabbit, at the end of a half hour the animal

¹ We wish to express our appreciation to Merck and Co. for furnishing the phenylcinchoninic acid and to Bilbuber Knoll Corporation for furnishing the metrazol used in these experiments

remains quiet and immobile, at an hour has lost spontaneity of movement and has reduced sensibility, at an hour and a half it is somnolent, and brusque movements appear in the hind legs, at 2 hours flaccid paralysis ensues, at 4 hours the animal begins to recover (4). In massive doses, 500-800 mg. per kilo, convulsions have been observed. The immediate toxic effects have been attributed to its action on the nervous system and the toxicity is said to be due to the adjunction of the phenyl group to the cinchoninic acid (5). After small doses, the blood pressure momentarily falls then rises; the pulse rate at first increases then diminishes; after section of the vagi the rate again increases; the respirations at first diminish then increase (4).

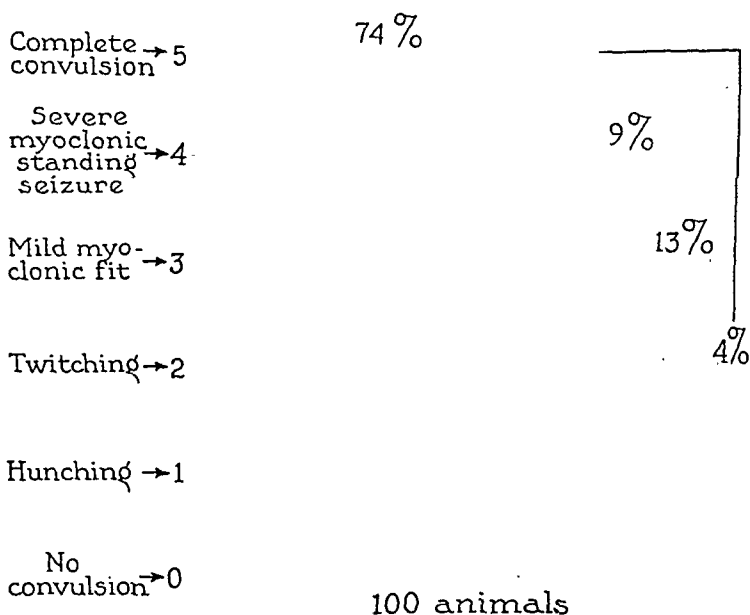


FIG. 1. PERCENTAGE DISTRIBUTION OF DEGREE OF SEVERITY OF CONVULSIONS IN 100 ANIMALS WHICH RECEIVED AN INTRAVENOUS INJECTION OF 13.2 MG. OF METRAZOL PER KILO

In our experiments the intravenous injection of from 88-220 mg. per kilo was not followed by any observable motor or sensory disturbance or somnolence. When 5 minutes following the intravenous injection of from 88 to 132 mg. per kilo, metrazol in doses of 13.2 mg. per kilo was injected intravenously into rabbits, in 50 rabbits so treated there ensued no complete convulsions (no. 5), no severe standing myoclonic seizures (no. 4), 10 (20 per cent) of mild standing myoclonic seizures, 7 (14 per cent) of seizures of lesser severity of this type (no. 3), 12 (24 per cent) of instances of only twitches, (no. 2) 4 (8 per cent) of hunchings (no. 1) and 17 with no motor manifestations (fig. 2). This may be contrasted with the occurrence of 74 per cent of complete convulsions in control groups.

Ten rabbits were fed phenylcinchoninic acid by mouth in doses 220 mg. per day per kilo for 5 days. Four of the animals died. When a minimal convulsant dose of metrazol was injected intravenously into the remaining 6, in only one was a complete convulsion observed, in the others only twitching of the ears and hunching. On the other hand, in 6 control animals 5 complete convulsions ensued.

The anticonvulsant property remains active an hour after intravenous injection of phenylcinchoninic acid. Thus at 15 and 30 minutes only slight twitches resulted; at 45 minutes only hunching; at an hour, of 11 animals, only

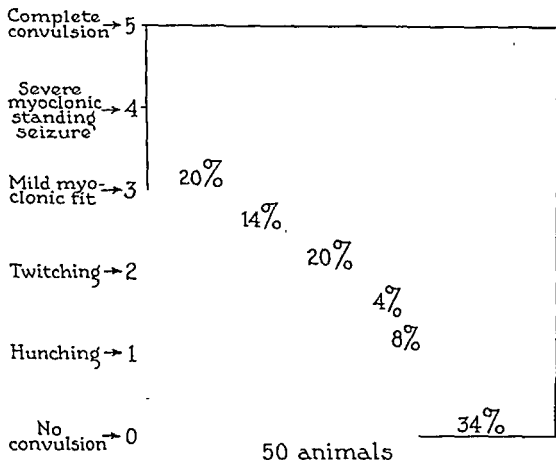


FIG. 2. PERCENTAGE DISTRIBUTION OF DEGREE OF SEVERITY OF CONVULSIONS IN 50 ANIMALS WHICH RECEIVED AN INTRAVENOUS INJECTION OF 88-132 MG. OF PHENYLCINCHONINIC ACID PER KILO, 5 MINUTES PREVIOUS TO THE INJECTION OF A MINIMAL CONVULSANT DOSE OF METRAZOL.

3 had complete convulsions, 1 a severe standing seizure, 3 only twitches and 4 no motor disturbance of any nature.

Phenylcinchoninic acid did not prevent the convulsions ensuing from intravenous or subcutaneous injections of minimal convulsant doses of picrotoxin or thujone. In 10 animals studied, neocinchophen (6-methyl-2-phenylquinoline-4-carboxylic acid ethyl ester) did not prevent metrazol convulsions.

The mode of action by which phenylcinchoninic acid prevents metrazol convulsions remains to be discovered. A direct chemical antagonism was excluded by the failure to prevent convulsions when phenylcinchoninic acid and its derivatives were first mixed with the metrazol and injected intravenously.

Since we found that phenylcinchoninic acid did not prevent convulsions produced by picrotoxin or thujone or the passage of alternating current through the head, we do not believe that the anticonvulsant action is the result of any specific depressant effect upon the brain stem, upon which medullary convulsants as camphor and picrotoxin alike exert their chief effect; or to a general narcotic action. The possibility that phenylcinchoninic acid may play a part in the detoxication of metrazol suggests further study.

CONCLUSION

The administration of suitable doses of phenylcinchoninic acid, intravenously or by mouth significantly reduces the severity or prevents the occurrence of convulsions after the intravenous administration of convulsant doses of metrazol.

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DETERMINATION OF ETHYL ALCOHOL WITH THE PHOTOELECTRIC COLORIMETER¹

HENRY NEWMAN AND MASON ABRAMSON²

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Six years ago Newman (1) described a simple, reliable method for the determination of ethyl alcohol in body fluids. The purpose of the present communication is to report minor improvements in technic, as well as the application of photoelectric colorimetry to the procedure.

In brief, the determination depends on vacuum distillation of the alcohol in the sample into an oxidizing solution consisting of potassium dichromate in strong sulphuric acid, with colorimetric determination of the amount of dichromate reduced. Rather than dwelling on the differences between this and the original method, we will describe the procedure as now used.

The only reagent necessary is a solution of potassium dichromate in equal parts of distilled water and concentrated sulphuric acid. The strength of dichromate used will depend on the range of alcohol concentrations to be covered; N/10 will be adequate for concentrations up to 300 mgm per 100 cc. when 1.0 cc. samples are used. The solution need not be standardized.

Sufficient anhydrous sodium sulphate is placed in a 25 cc. Erlenmeyer flask to cover its bottom. Onto this is accurately pipetted 1.0 cc. of the sample to be examined. The flask is closed with a rubber stopper bearing an inlet tube with a stop-cock, and an L-shaped outlet tube, expanded in its vertical portion. This is connected by means of rubber tubing with the inlet of the receiving vessel, which is a Folin blood sugar tube graduated at 25.0 cc. into which has been pipetted exactly 3.0 cc. of the dichromate solution. This tube is closed by a rubber stopper bearing the inlet tube, which reaches almost to its bottom and terminates in a small aperture to decrease bubble size, and the outlet tube which ends flush with the stopper and connects through a stop-cock with the vacuum line. A vane type vacuum pump, capable of maintaining a vacuum of 0.02 mm., is used, connected to a manifold whereby 5 determinations may be run simultaneously. The sample flasks rest in a water bath maintained between 50° and 55° C., and a tubular electric light running alongside the delivery tubes maintains the temperature of this region somewhat above that of the room, and discourages condensation of alcohol in the tubes when the laboratory temperature is low.

When the apparatus is connected up as described, the stop-cock on the inlet tube of the sample flask is closed and that to the vacuum slowly opened. Distillation is allowed to proceed for approximately 20 minutes, when the stop-cock to the vacuum is closed, and that on the inlet tube slowly opened. The tube containing the oxidizing solution is then removed, its stopper and inlet tube carefully washed into it, and the volume made up to the 25 cc. mark with distilled water. The contents are thoroughly mixed by inversion, and a portion placed in the cuvette of the colorimeter, where the transmittance of light of a wavelength of 480 millimicrons is determined, the comparison solution being obtained from a blank determination in which the sample consisted of distilled water. The concentration

¹ Supported in part by a grant from the Rockefeller Fluid Research Fund of the Stanford University School of Medicine.

² From the Department of Medicine, Stanford University School of Medicine.

of alcohol in the sample is then read off a calibration curve, prepared by plotting the results of the determination of a number of known concentrations of alcohol throughout the range on semi-logarithmic paper.

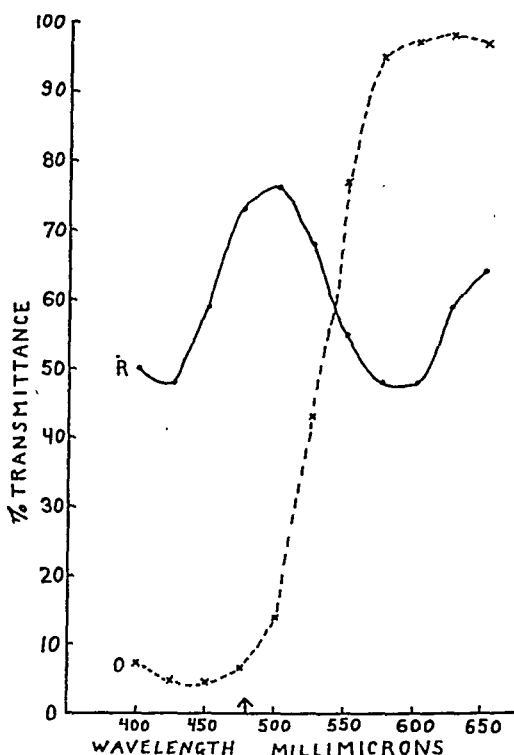


FIG. 1. TRANSMITTANCE CURVES OF THE OXIDIZING SOLUTION, O, AND THE SAME SOLUTION AFTER REDUCTION, R, DETERMINED WITH THE SPECTROPHOTOMETER

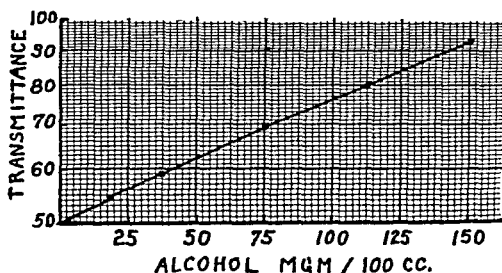


FIG. 2. CALIBRATION CURVE FOR APPROXIMATELY N/20 OXIDIZING SOLUTION, WITH LIGHT OF A WAVE-LENGTH OF 480 MILLIMICRONS

It will be noted that it departs to some extent from a straight line. Since the unknown transmits more light than the standard, the setting for the standard was taken at 50 per cent. Thus actual percentage transmission must be calculated by multiplying the scale by 2.

Comment. The changes in the procedure, notably the increase in the distillation temperature and the warming of the delivery tube, have been found to eliminate the occasional aberrant results found with the original method. They have, however, not appreciably improved the probable error of the method as previously reported (1), which was not over 3.5 per cent for concentrations over 25 mgm. per 100 cc.

Substitution of colorimetric for iodometric determination of the remaining dichromate has been accomplished without any sacrifice of accuracy. The apparatus used is a Coleman Model 11 Spectrophotometer. The procedure is complicated by the fact that the reduction products of the dichromate are not colorless, as can be appreciated from the spectral transmittance curve in figure 1, which also shows the curve for the unreduced dichromate. Largely by a process of trial and error, it was found that a wavelength of 480 millimicrons, at which the transmittance of the reduction products is maximal and that of the dichromate has not yet risen significantly, is the most satisfactory, and that the best reference solution at this wavelength is the unreduced dichromate. Because the reference solution transmits less light than the unknown, it is necessary with the Coleman instrument to use 30 per cent transmittance as a reference with N/10 and 50 per cent with N/20. A calibration curve obtained with the latter is shown in figure 2. As is readily made out, this is not a straight line, indicating that Beer's law does not strictly apply. This necessitates the experimental determination of a number of points on the curve, which is done by running in duplicate or triplicate alcohol solutions of known concentration, made up from absolute alcohol by serial dilution. Since the dichromate solution keeps indefinitely, it may be made up in large amount, and the calibration curve remains valid until the solution is exhausted.

The procedure should be readily applicable to other types of photoelectric colorimeter. The filter used should have its maximal transmission at about 480 millimicrons. The great advantage of the colorimetric procedure over the iodometric lies in the lack of necessity for standardization of solutions, and expediency in reading the colorimeter compared with titration. Careful quantitative technique is still essential to consistent results. There is no advantage to be gained by having a battery of more than 5 sets of apparatus, with which little difficulty is experienced in running ten determinations an hour.

The simplicity of the procedure recommends it for medico-legal work, while its accuracy is adequate for research purposes.

SUMMARY

The concentration of alcohol in solutions may be accurately determined by vacuum distillation of the sample off of anhydrous sodium sulphate at a temperature of 50 to 55 degrees C. into a solution of potassium dichromate in strong sulphuric acid, and the determination of the amount of dichromate reduced by photoelectric colorimetry.

REFERENCE

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PHARMACOLOGY AND CHEMISTRY OF SUBSTANCES WITH CARDIAC ACTIVITY

I. EFFECT OF UNSATURATED LACTONES ON THE ISOLATED FROG HEART¹

O. KRAYER, R. MENDEZ, E. MOISSET DE ESPANÉS² AND R. P. LINSTEAD

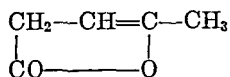
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of the Department of Chemistry, Harvard University*

Received for publication December 27, 1941

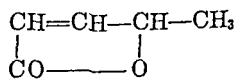
The general object of the series of papers of which this is the first is to investigate the relationship between the chemical structure and pharmacological activity of compounds which have a digitalis-like action on the heart. The most numerous and important group of these substances are the cardiac glycosides, which are related in chemical structure to the sterols. This group includes the active principles of digitalis, strophanthin, and squill. All the members of the group contain an unsaturated lactone ring in the molecule, and it is well established that modification of this structural feature leads to a great diminution or even extinction of the activity. It therefore becomes of considerable interest to examine how far it is possible to reproduce the cardiac activity of the natural unsaturated lactones in synthetic compounds containing the same apparently essential structural feature. The unsaturated lactones are not a particularly accessible group of compounds and until recently only a comparatively small number had been synthesized and investigated chemically. Pharmacological examination of some of the simpler members of the group had failed to reveal any cardiac activity (1).

It is shown in the present paper that, under suitable experimental conditions, unsaturated lactones of quite simple structure have a definite and reproducible pharmacological effect on the frog heart. The same general result has been reached simultaneously and independently by Chen, Elderfield, and their collaborators, using a different technique (see the following paper). We are indebted to Drs. Chen and Elderfield for their friendly coöperation in this matter.

In pursuance of the general objective of our work a considerable number of unsaturated lactones have been prepared. In the present paper we describe the results obtained from only four of these, whose effects on the frog heart have been investigated by the methods described in this paper. These are the substances:



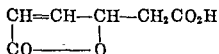
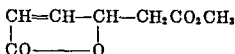
(I) $\beta\gamma$ -angelicalactone



(II) $\alpha\beta$ -angelicalactone

¹ This work was done under the auspices of the University Committee on Pharmacotherapy.

² Fellow of the Rockefeller Foundation.

(III) Crotonolactone γ -acetic acid

(IV) Methyl ester of (III)

The two angelicalactones are well known substances, and were prepared by standard methods. To avoid any ambiguity in nomenclature we shall refer to them in the manner shown above, in which the prefix indicates the position of the double bond. The early literature (2) named the $\beta\gamma$ - and $\alpha\beta$ -unsaturated lactones the " α -" and " β -" lactones, respectively. These arbitrary and confusing prefixes had better be discarded.

The other two compounds are new. The lactonic acid (III) is a carboxy derivative of $\alpha\beta$ -angelicalactone, and (IV) is its methyl ester. The lactonic acid was prepared from *cis-cis*-muconic acid (3) by lactonization with sulfuric acid. It is a beautifully crystalline solid which melts at 110–112°C. It was proved to contain one double bond, one free carboxyl group, and a lactone ring. The

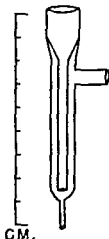


Fig. 1

position of the double bond was shown by its chemical reactions and absorption spectrum. The methyl ester could be obtained from it either by means of diazomethane or through the acid chloride. It is a crystalline solid melting at 31°. The synthesis and chemical investigation of these substances were mainly carried out by Mr. Bernard A. Orkin. Details will be published elsewhere.

METHODS. Our experiments were carried out on frogs (mostly male) of the species *Rana pipiens* in October, November, and December, 1941. The isolated frog hearts were prepared according to Straub but we used the straight Fühner cannula. The amount of fluid in the cannula was 1.5 cc.

In a series of experiments we used a cannula illustrated in figure 1.³ The content of the cannula was continuously replaced by dripping fresh solution into it from a Mariotte bottle. A constant rate of replacement was necessary in order to get consistent results. We chose

³ This cannula was devised and made by Mr. Henry George, Laboratory Assistant of the Department of Pharmacology.

arbitrarily a rate of replacement of 2 to 2.5 cc. per minute for the whole of this series of experiments.

In another series of experiments, in order to study the action of the substances upon the output of the heart, we perfused the frog heart from the venous side using the method described by Bülbiring (4). The perfusion fluids were placed in Mariotte bottles and the output of the heart was measured from a cannula tied into the left aorta. The venous supply was arranged so that the ventricle worked with its maximal stroke volume. The opening of the outflow cannula was kept 12 to 15 cm. above the level of the heart.

The perfusion fluid had the following composition: NaCl, 0.65 per cent; KCl, 0.014 per cent; CaCl_2 , 0.011 per cent; NaHCO_3 , 0.02 per cent. The solution poor in calcium ions which was used for some experiments differed from this in that it contained only 0.0055 per cent CaCl_2 .

Ethyl alcohol was used as a solvent for the lactones and the lactonic ester. A 5 per cent stock solution was appropriately diluted with the salt solution prior to the experiment. More concentrated stock solutions were found to show precipitation when the salt solution was added. The lactic acid was sufficiently soluble in the salt solution. As Trendelenburg already observed, alcohol may modify the action of cardioactive substances (5). In control experiments we have convinced ourselves that none of the characteristic effects ascribed to the lactones could have been produced by the alcohol in the concentrations which we employed.

The hydrogen-ion concentration of all solutions was adjusted to that of the normal salt solution and was kept between pH 7.5 and 7.8. The frogs were kept at room temperature for at least 24 hours prior to the experiment. All the experiments were conducted at a temperature between 23° and 25°C.

RESULTS. 1. *The effect of $\beta\gamma$ -angelicalactone.* A concentration of 1:50,000 of $\beta\gamma$ -angelicalactone, given once into the Fühner cannula, caused an increase in amplitude of contraction of the isolated heart with little or no change in heart rate. The effect lasted for several minutes. Higher concentrations showed a similar effect but of longer duration; for instance, 1:10,000 was effective for more than one-half hour; below 1:5,000 the period of the increase in amplitude was preceded by a short period during which the amplitudes were smaller. This depressing effect was probably due in part to the alcohol concentration of 0.4 vol. per cent or more. With a concentration as high as 1:1,000 (alcohol concentration 2 per cent) extra-systoles and groupings of contractions were not infrequent; auriculo-ventricular and sino-auricular block were sometimes observed. The effect of a single administration of the lactone could be reversed by washing.

When the solution of the $\beta\gamma$ -angelicalactone was replaced the increase in amplitude became more pronounced; if the replacement was repeated often enough with a suitable concentration the ventricle dilated less and less during the diastole, and the heart eventually came to an irreversible systolic standstill (fig. 2). In the experiment of figure 2 a concentration of 1:50,000 was used and replaced every five minutes. The increase in amplitude became apparent during the second five-minute period, and systolic standstill was reached 3 hours and 25 minutes after the first administration.

With the same concentration, the time required to achieve systolic standstill was, up to a certain limit, inversely proportional to the number of replacements. Figure 3 shows this relation when a concentration of 1:50,000 was used and when the time interval between successive replacements ranged from 1 to 60 minutes.

The relation between concentration and speed of action could be studied more easily if a continuous replacement of the fluid was made through the cannula described in figure 1. The result of such an experiment is shown in figure 4

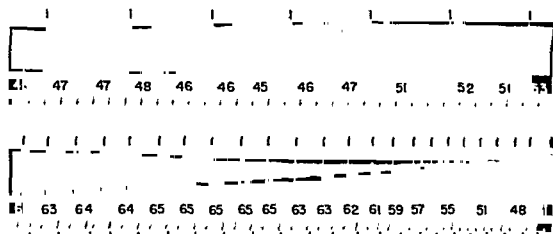


FIG. 2. FROG HEART (STRAUB), FÜHNER CANNULA

Between upper and

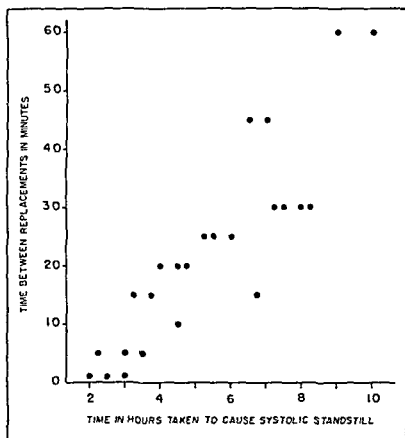


FIG. 3. FROG HEART (STRAUB) FÜHNER CANNULA

Relation between frequency of replacements of a solution of $\beta\gamma$ -angelicalactone 1:50,000, and the time taken to produce systolic standstill.

with a concentration of 1:25,000. The first effect was an increase in amplitude appearing after a latent period of about two minutes. Twelve minutes after the beginning of the administration of $\beta\gamma$ -angelicalactone the diastolic relaxation became less complete, and 20 minutes after the beginning of the administration the systolic height of contraction diminished somewhat. The amplitude of the ventricular contractions decreased steadily and the ventricle stopped in complete systole after 42 minutes.

As can be seen from figure 7, this technique reveals a characteristic relation between the concentration of the angelicalactone and the time required to cause stoppage of the ventricle. A concentration of 1:1,000 (0.01 molar) brought about systolic standstill of the heart within 24 minutes, while 1:1 million (0.00001 molar) was approximately the limit of the concentration causing systolic standstill. In three experiments with this concentration one heart stopped in systole after 363 minutes, one after 570 minutes, and one had not stopped after 24 hours.

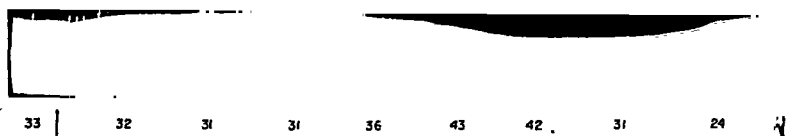


FIG. 4. FROG HEART. CANNULA OF FIG. 1

The action of $\beta\gamma$ -angelicalactone. Continuous replacement of solution. At sign start of the administration of the $\beta\gamma$ -lactone 1:25,000. Rate of replacement: 2.4 cc. per minute. The figures indicate heart rate per minute. A means auricular rate. Time in 30-second intervals.

Changes in rate with either of the two methods were not uniform. As a rule there was an increase in rate when the "systolic" effect became pronounced. Usually this was particularly marked in hearts with a low normal rate. The rate then dropped again when the ventricle approached the complete systolic position. The auricular rate was regular unless very high concentrations were used, and as a rule the auricles continued beating for many hours after the ventricle had stopped. Often the auricular rate (after the systolic standstill of the ventricle was reached) corresponded to the normal rate of the heart. Sometimes it was lower, as in figure 4. Occasional extrasystoles have sometimes been recorded, but no atrio-ventricular block has been observed in any of the experiments which were made with the cannula of figure 1.

If the $\beta\gamma$ -angelicalactone was given to a heart which had been beating with normal perfusion fluid for some time and showed a decrease in amplitude as compared to the initial amplitude, the increase in amplitude was much more pronounced than in a heart freshly prepared. The same was observed when concentrations of alcohol up to 1 per cent had acted upon the heart and had markedly decreased the amplitude of the ventricular excursion. If a heart was beating on the Fühner cannula with salt solution poor in calcium ions and the

characteristic decrease in amplitude had been established, the administration of angelicalactone either restored a normal amplitude or at least markedly increased it.

That this action of the lactone improved the heart and enabled it to increase its stroke volume could be demonstrated by perfusing the heart from the venous side and measuring its output.⁴ Figure 5 represents such an experiment. With normal perfusion fluid the output of this heart ranged between 10 and 7.5 cc. per minute; the rate decreased from 59 to 43 per minute during the period previous to the shift to the calcium-poor solution. The stroke volume, however, did not change; it ranged between 98 per cent and 102 per cent of the average normal stroke volume of 0.177 cc. Perfusion with the solution poor in calcium

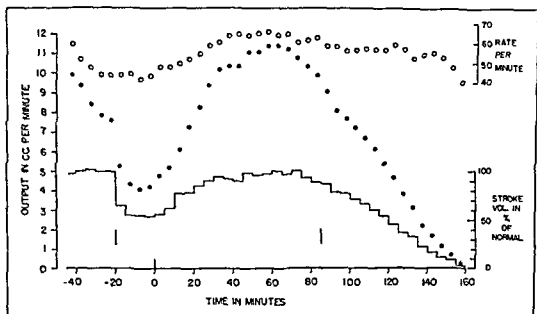


FIG. 5 FROG HEART

did not further change the rate, but the output dropped sharply to between 4 and 4.3 cc. per minute, a decrease in stroke volume to 54 to 56 per cent of the normal. After the perfusion with $\beta\gamma$ -angelicalactone 1:500,000 in calcium-poor solution had started, the output increased within two minutes, and within one-half hour reached 10 cc. per minute, the highest value observed in the preliminary control period. For about one hour after this the output stayed between 10 and 11.6 cc. per minute, then began to drop, and gradually decreased to reach zero $1\frac{1}{2}$ hours later. During the initial phase of the increase in output, which lasted for about 45 minutes, the heart rate increased gradually from 43 to 65, while simultaneously the stroke volume rose to 90 per cent of the normal volume. In

⁴ Some of these experiments were made by Dr. G. Montes.

the second phase, which lasted about 30 minutes, no further change in rate occurred, while the stroke volume reached normal values. The output, 10.5 to 11.6 cc. per minute, was above the normal because of an increase in rate. In the third phase the output dropped gradually to less than 1 cc. per minute. As there was only a decrease in rate from 65 to 52 during this period, a marked diminution of the stroke volume was responsible for the decrease in output. At the beginning of this phase the heart assumed a more systolic position and subsequently lost more and more its ability to dilate during the diastolic period. Eventually the ventricle ceased to dilate and the heart gave the familiar appearance of the frog heart *in situ* in systolic standstill, with the auricles full of fluid and still beating but unable to empty themselves into the ventricle. During this terminal phase the auricle beat regularly at a rate of 40 per minute. If larger doses were given (1:200,000 to 1:50,000) the period during which the heart was able to work with undiminished stroke volume was cut short much sooner by the ensuing toxic action. With a concentration of 1:50,000, for instance, this period lasted only about 5 minutes and the ventricle stopped in systole 30 minutes after the beginning of the administration of the $\beta\gamma$ -angelicalactone.

2. *The effect of $\alpha\beta$ -angelicalactone, lactonic acid (III), and lactonic ester (IV).* The $\alpha\beta$ -angelicalactone had qualitatively the same action as the $\beta\gamma$ -lactone. Like the latter it produced an increase in the amplitude of contraction which was particularly pronounced if the heart was hypodynamic. Figure 6 illustrates this action on a heart beating with a solution poor in calcium. When administered the first time (see upper curve of fig. 6) the effect upon the amplitude of contraction became apparent after 1 minute, and full effect was obtained within 15 minutes. To subsequent administrations the heart did not react so quickly, and several replacements of the same concentration were sometimes necessary to obtain a marked effect. (See lower curve of fig. 6).

As with $\beta\gamma$ -lactone, systolic standstill of the heart could be obtained if the $\alpha\beta$ -lactone solution was replaced often enough or administered long enough to the heart, but there was a marked quantitative difference between the $\beta\gamma$ -lactone and the $\alpha\beta$ -lactone. This is obvious from a comparison of the concentration-time relation for the systolic standstill as shown in figure 7. The $\alpha\beta$ -lactone is considerably less potent than the $\beta\gamma$ -lactone.

Figure 7 also shows the time concentration curves for the angelicalactones as compared with digitoxin and *g*-strophanthin using the same method. Taking the limit molar concentration of $\beta\gamma$ -lactone capable of producing systolic standstill as 1, that of the $\alpha\beta$ -lactone is equal to 10, while those of digitoxin and *g*-strophanthin would be of the order of 0.026 and 0.017 respectively. This approximate relation of 50 to 1, if digitoxin is compared with the $\beta\gamma$ -angelicalactone in this fashion, does not hold if the glycoside and the lactone are compared in regard to the concentration necessary to produce systolic standstill in a short period of time, for, as can be seen from figure 7, the concentration of $\beta\gamma$ -lactone capable of producing systolic standstill in approximately 20 minutes is about 500 times as great as that of digitoxin.

The higher activity of the $\beta\gamma$ -lactone takes on a special interest in relation to the recent discussion of the position of the double bond in the highly active

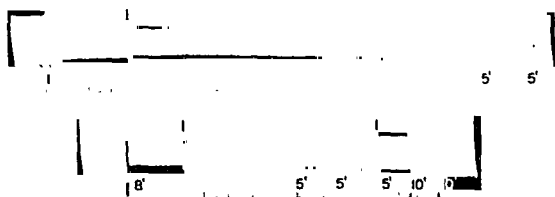


FIG. 6. FROG HEART (STRAUB), FÜHNER CANNULA

At signs below the tracings the normal perfusion fluid was exchanged for the solution poor in calcium. At the signs above the tracings the calcium-poor salt solution was replaced by a calcium-poor solution containing 1:50,000 $\alpha\beta$ -angelicalactone. The figures indicate periods in minutes when the drum was arrested. Time in 10-second intervals.

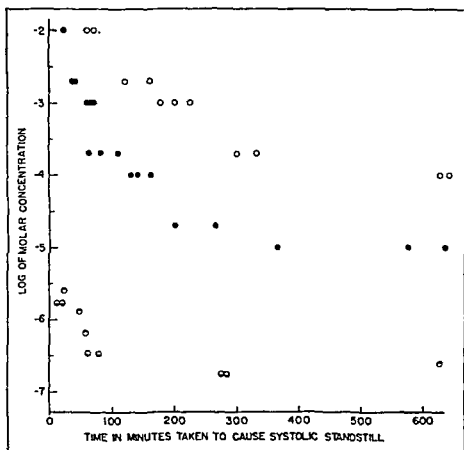
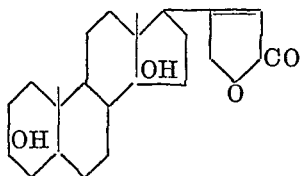


FIG. 7. FROG HEART. CANNULA OF FIG. 1

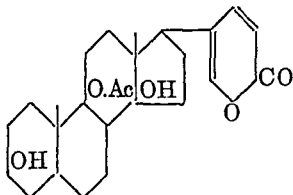
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pared in our own laboratory according to Gilmour's modification (15) of Thiele, Tischbein, and Lossow's method (16). It therefore appears desirable to examine pharmacologically other unsaturated lactones that have become available in the course of our chemical investigation.

The list is composed of twenty-seven compounds as enumerated in table 1. Owing to the ease of confusion in nomenclature, their structural formulas have been appended. The polymer of β,γ -angelica lactone, prepared and furnished by Professor Marvel (17), and nepetalactone, isolated from the volatile oil of catnip and generously supplied by Professor S. M. McElvain (18), Department of Chemistry, University of Wisconsin, Madison, were included. The last four products in table 1 were made according to the methods of Knoevenagel (19) and Linch (20) without alteration. Briefly, the entire group represents derivatives of 5- and 6-member ring compounds, simulating the side chains of many aglycones and toad poison principles, as exemplified below:



Digitoxigenin



Bufotalin

RESULTS IN FROGS. The simplest test for these substances can be carried out in frogs by injection into the abdominal lymph sac. The well-known 1-hour method is satisfactory. In our experiments, the temperature of the bath varied from 22.5° to 28°C., depending on the atmospheric temperature. Over 90 per cent of the frogs were male. The weight of the entire group of animals lay between 12 and 37.8 gm., and that of the majority was in the neighborhood of 20 gm.

The results were recorded as scrupulously as possible. Only typical systoles were marked positive. If the ventricle was in partial systole at the end of an hour, it was considered negative. When a compound was found to be active, the test was repeated by not only the same investigator, but also another observer familiar with the procedures, for the sake of confirmation.

Most of the compounds studied were partly soluble in water, requiring ethyl alcohol for complete solution. Particular care was exercised to use the minimal volume of alcohol, for control experiments showed that 1 to 2 cc. of 95 per cent alcohol injected into the lymph sacs of frogs weighing 24 to 25 gm. occasionally caused systolic standstill in an hour. Besides, the depressant action of alcohol must also be considered as an undesirable complication.

At the beginning of the investigation the 16-hour overnight method was tried. More positive results were obtained. These proved to be non-specific or false positives, for lethal doses of ephedrine and a derivative of barbituric acid, both unrelated to cardiac substances, also caused typical systolic stoppage. For the

TABLE 1
The formulas of lactones investigated


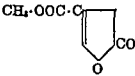
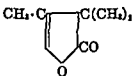
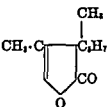
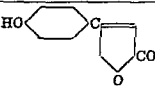
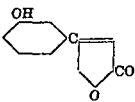
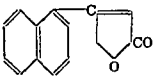
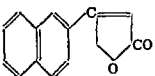
COMPOUND NUMBER	CHEMICAL NAME AND REFERENCE FOR METHOD OF PREPARATION	STRUCTURAL FORMULA
1	β,γ -Angelica lactone (15) (16)	
2	Polymer of β,γ -angelica lactone (17)	
3	Methyl aconate (21)	
4	Blaise and Courtot lactone (22)	
5	Nepetalactone (18)	
6	β - <i>p</i> -Hydroxy-phenyl- Δ^5 - δ -butenolide (23)	
7	β -(<i>m</i> -Hydroxy-phenyl)- Δ^5 - δ -butenolide (23)	
8	β -(α -Naphthyl)- Δ^5 - δ -butenolide (23)	
9	β -(β -Naphthyl)- Δ^5 - δ -butenolide (5)	

TABLE 1—Continued

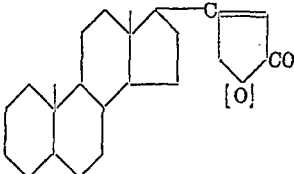

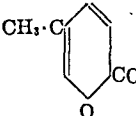
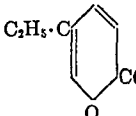
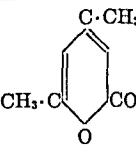
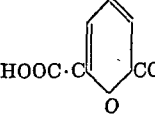
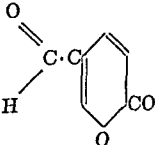
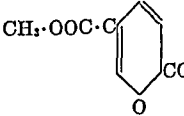
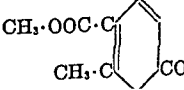
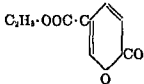
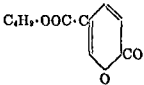
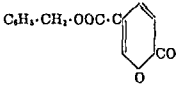
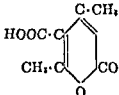
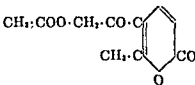
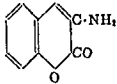
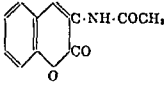
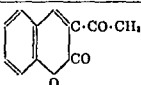
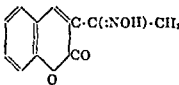
COMPOUND NUMBER	CHEMICAL NAME AND REFERENCE FOR METHOD OF PREPARATION	STRUCTURAL FORMULA
10	Lactone of 21-hydroxy- $\Delta^{20,22}$ -nor- cholenic acid (10)	
11	α -Pyrone (8)	
12	5-Methyl- α -pyrone (8)	
13	5-Ethyl- α -pyrone (8)	
14	4,6-Dimethyl- α -pyrone (24)	
15	α -Pyrone-6-carboxylic acid (25)	
16	Coumaly aldehyde (23)	
17	Methyl coumalate (9) (26)	
18	6-Methyl-methyl coumalate (9)	

TABLE 1—Concluded

COMPOUND NUMBER	CHEMICAL NAME AND REFERENCE FOR METHOD OF PREPARATION	STRUCTURAL FORMULA
19	Ethyl coumalate (26) (27)	
20	<i>iso</i> -Butyl coumalate (23)	
21	Benzyl coumalate (23)	
22	4,6-Dimethyl- α -pyrone-5-carboxylic acid (24)	
23	6-Methyl-5-(ω -acetoxy)-aceto- α -pyrone (9)	
24	3-Amino-coumarin (20)	
25	3-Acetyl-amino-coumarin (20)	
26	3-Acetyl-coumarin (19)	
27	Oxime of 3-acetyl-coumarin (20)	

study of new compounds, this method is unsuitable. It was therefore quickly abandoned in our laboratory.

TABLE 2
Activity of 5 compounds in frogs

COMPOUND	HIGHEST CONCENTRATION USED	ALCOHOL BY VOLUME	DOSE	NUMBER OF FROGS INJECTED	NUMBER OF HEARTS IN SYSTOLE
	<i>per cent</i>	<i>per cent</i>	<i>mgm. per gm.</i>		
β, γ -Angelica lactone	10	23.75	1.0	10	0
			1.5	10	0
			1.75	10	0
			2.0	20	2
			2.25	9	4
			2.5	18	1
			3.0	23	9
			3.5	13	9
			4.0	15	12
			5.0	11	9
β -(β -Naphthyl)- $\Delta^{\alpha, \beta}$ -butenolide	< 4 (satur- ated)	95.0	<1.35	1	0
			<1.66	1	1
			<1.73	1	1
			<2.08	1	1
Lactone of 21 -hydroxy- $\Delta^{20, 22}$ -nor- cholenic acid	0.4	66.5	0.03-0.07	4	0
			0.12	1	0
			0.14	1	0
			0.21	2	1
			0.25	1	1
			0.38	1	1
Methyl coumalate	5	28.5	0.3	5	1
			0.4	5	1
			0.5	20	5
			0.6	25	10
			0.7	25	14
			0.8	25	14
			0.9	25	16
			1.0	25	16
			1.1	10	5
			1.2	10	1
Ethyl coumalate	5	28.5	0.5	5	0
			0.6	5	1
			0.7	5	2
			0.8	5	2
			0.9	5	3
			1.0	5	4

Out of 27 compounds, 4 showed definite, and one suggestive activity by the 1-hour frog method. The results are summarized in table 2. Although the

potency of these substances does not approach that of the aglycones, strophanthidin, digoxigenin, digitoxigenin, and calotropagenin (28), at least two of them are superior in action to the natural glycoside uzarin (14). On perfusion into the inferior vena cava, the systolic contracture of the ventricle, as shown in figure 1, was not as extreme as that familiarly induced by ouabain. The tracings are more like those produced by perfusion with uzarin and certain aglycones. Direct inspection of the heart an hour after injection of a sufficient dose, however, revealed an unmistakable picture of the effect, the ventricle being white and tightly contracted and the auricles widely dilated.

With β, γ -angelica lactone, 6 separate assays were carried out on two different samples, one furnished by Professor Marvel and the other prepared by ourselves. The results show a consistent trend; consequently, they are combined in table 2. The median systolic dose computed from this set of data is 3.23 ± 0.15 mgm. per gm. The doses employed in a previous study (14) were less than 2 mgm. per gm., which accounts for the discrepancy.



FIGURE 1. THE ACTION OF β, γ -ANGELICA LACTONE (LABELLED α -ANGELICA LACTONE) AND ETHYL COUMALATE ON THE FROG'S HEART BY PERFUSION INTO THE INFERIOR VENA CAVA

The results of β -(β -naphthyl)- $\Delta^{\alpha, \beta}$ -butenolide are less certain, because they are complicated by the high content of alcohol. However, according to our experience, less false positives occur than with alcohol alone. It was therefore proposed that the product had a suggestive activity.

With the lactone of 21-hydroxy- $\Delta^{20, 22}$ -norcholenic acid, claim for its activity can probably be made with greater security, because none of the five control animals receiving amounts of alcohol equivalent to those present in the highest dose of the drug developed systolic standstill. Owing to the small quantity available, no extensive studies were carried out. The positive finding in this case, however, is of particular interest, because the compound having a steroid ring is closest to the structure of aglycones in our series.

Methyl coumalate has a definite action on the frog's heart. The same was observed by Professor McKeen Cattell (29), Cornell University Medical College, New York City. Results from 6 assays, combined in table 2, indicate that the optimal doses lie between 0.7 and 1 mgm. per gm. The reason why quantities larger than 1 mgm. per gm. became less effective must be left for future ex-

planation. Ethyl coumalate, like the methyl derivative, produced systolic arrest. The results are comparatively more consistent, permitting the computation of the median systolic dose, 0.81 ± 0.06 mgm. per gm.

The remaining 22 compounds proved to be inactive as shown in table 3. This conclusion, however, should be conditioned by the fact that 12 of them were tested in doses less than 1 mgm. per gm. Such small dosage was necessitated by either the limited solubility of the compound or the small amount of the material available.

TABLE 3
Compounds showing no activity in the doses employed

COMPOUND NUMBER	HIGHEST CONCENTRATION USED	ALCOHOL BY VOLUME	NUMBER OF FROGS INJECTED	LARGEST DOSE EMPLOYED
	<i>per cent</i>	<i>per cent</i>		<i>mgm. per gm.</i>
2	4	23.75	5	4.60
3	4	28.5	9	5.19
4	4	38.0	5	4.91
5	4	52.25	8	5.63
6	1	85.5	5	0.99
7	0.5	47.0	8	0.36
8	0.5	57.0	8	0.38
11	3.5	14.2	20	1.79
12	4	0	20	1.87
13	3	28.5	20	1.38
14	1	9.5	10	0.54
15	1	14.25	10	0.59
16	5	47.5	5	4.88
18	1	14.25	10	0.59
20	5	57.0	5	5.21
21	5	85.5	3	1.67
22	1	14.25	10	0.55
23	1	14.25	10	0.45
24	0.5	76.0	8	0.41
25	0.5	57.0	8	0.36
26	0.5	66.5	13	0.51
27	0.5	57.0	13	0.49

RESULTS IN CATS. No preliminary investigation on a digitalis-like drug can be said to be adequate until a study in warm-blooded animals such as the cat has been made. The latter gives rise to additional information not easily obtainable in frogs. First of all, there are characteristic changes on the electrocardiogram. Secondly, all the known glycosides when rapidly injected by vein raise arterial blood pressure followed by a sudden circulatory collapse if a lethal dose has been administered; and thirdly, they cause nausea and vomiting in non-anesthetized cats after intravenous injection of suitable doses.

Two etherized cats were injected intravenously with a 4 per cent solution of β , γ -angelica lactone at the rate of 1 cc. per minute. An amount of 10.45 per cent alcohol was present. As customary in our previous studies (30) on cardiac

substances, electrocardiograms were obtained periodically during the course of injection. One animal succumbed to a dose of 617 mgm. per kgm., while the other survived one of 330 mgm. per kgm. With the exception of the end point, the results were similar. A few selected tracings from one animal are shown in figure 2. There occurred gradual slowing of heart rate, prolongation of

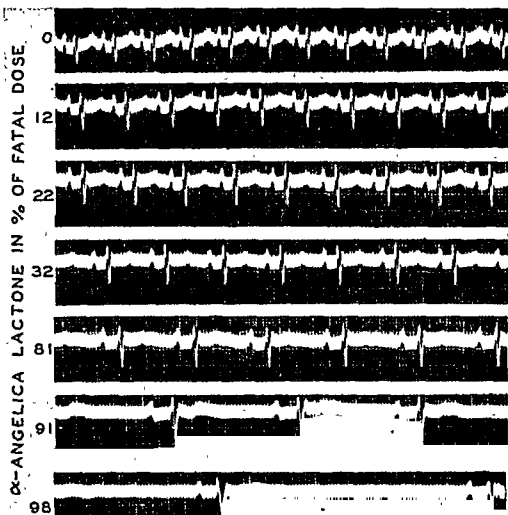


FIGURE 2. ACTION OF β,γ -ANGELICA LACTONE (LABELLED α -ANGELICA LACTONE) ON THE CAT'S HEART

Cat, male, weighing 2 596 kgm., was anesthetized by ether. β,γ -Angelica lactone in a 4 cin at the rate of 1 cc. per minute. A total 11, but only 7 selected tracings are shown and 0.1 second, and ordinates in 0.0001 and 1gm. per kgm.

P-R interval, and flattening of the *T*-wave. The reduction of heart rate may be partly attributed to alcohol. Conspicuously absent were ectopic rhythm, secondary tachycardia, and in the case of the cat receiving the larger dose, ventricular fibrillation at the point of death. As a whole, the electrocardiographic changes are not sufficient to constitute an unequivocal evidence for digitalis-like action. β,γ -Angelica lactone must be considered, therefore, devoid of such an action in cats.

Methyl coumalate in a 2 per cent solution was similarly administered in two cats at the rate of 4 cc. per minute. The alcoholic content was 13.25 per cent by volume. One animal died with a dose of 396 mgm. per kgm. while the other remained alive after 168 mgm. per kgm. Electrocardiographically, the *T*-wave became flattened, and later diphasic. In one case, it was ultimately inverted. There was a slight prolongation of the *P-R* interval. In the cat receiving the lethal dose, the *P*-wave dipped downward at a few places, and disappeared at the end. The heart rate progressively diminished from 214 to 37 per minute. In the other animal receiving the smaller dose, the sinus rhythm continued throughout, and the heart rate decreased from 191 to 155 followed by acceleration to 200 per minute.

One cat was employed for the electrocardiographic study during the injection of a 4 per cent solution of ethyl coumalate in 20.9 per cent alcohol by volume, at the rate of 2 cc. per minute. The quantity required to kill that animal was 385 mgm. per kgm. Death was not due to alcohol because in control animals it took twice the volume of the solvent to kill. Gradual slowing of the heart rate, slight lengthening of the *P-R* interval, and nodal rhythm occurred. The alterations caused by both methyl and ethyl coumalates lacked the appearance of ectopic rhythm, multiple foci of impulse formation, aberrant *QRS* complexes, secondary tachycardia, and terminal ventricular fibrillation, which have been consistently observed with numerous digitalis-like principles investigated in the same manner.

Five other cats, anesthetized with ether, were used for blood pressure studies. They weighed approximately 2.6 kgm. each. β,γ -Angelica lactone, methyl coumalate, or ethyl coumalate, in the dose (total) varying from 40 to 300 mgm., rapidly injected into the femoral vein, produced without exception a pure fall of blood pressure. Injections of the alcoholic solvent without the drug in the same volume resulted in a slight lowering of pressure, so that the effect observed was chiefly due to the medication.

β,γ -Angelica lactone was injected intravenously into three other non-anesthetized cats. A dose of 100 mgm. per kgm. caused convulsions but no emesis, one of 75 mgm. per kgm. was followed by salivation and defecation, while that of 50 mgm. per kgm. was ineffective. Methyl coumalate produced tonic convulsions in a cat with a dose of 75 mgm. per kgm., and tremor in another with that of 100 mgm. per kgm. Ethyl coumalate killed one cat with a dose of 100 mgm. per kgm., but 75 mgm. per kgm. in another caused prostration, followed by tremor, for over 2 hours. Again no vomiting occurred with either compound.

SUMMARY

1. Twenty-seven unsaturated lactones related to cardiac aglycones have been studied pharmacologically.

2. β,γ -Angelica lactone, the lactone of 21-hydroxy- $\Delta^{20,22}$ -norcholenic acid, methyl coumalate, and ethyl coumalate, cause systolic standstill of frogs' ventricles when adequate doses are injected into the ventral lymph sac. β -(β -Naphthyl)- $\Delta^{2,3}$ -butenolide has a suggestive action.

3. In cats, β , γ -angelica lactone and methyl and ethyl coumalates all produce a fall of blood pressure and fail to induce emesis. Electrocardiographically, no ectopic rhythm, multiple foci of impulse formation, various forms of aberrant QRS complexes, and terminal ventricular fibrillation occur as the lethal dose is slowly approached. The evidence in cats is therefore not indicative of any digitalis-like action of the three substances.

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THE ACTIVITY OF DERIVATIVES OF CURARE AS PREPARED IN LIQUID AMMONIA

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The use of curare as a drug for clinical and experimental purposes has been increasing in recent years (1). One deterrent to its wider use, however, has been its lack of chemical uniformity. Several workers have concerned themselves with the purification and fractionation of curare, among whom King (2) is outstanding. Much of this work, which is costly and time-consuming, has involved the isolation of individual alkaloids which make up part of the curare complex. It seemed to us that the development of some method of purification that would improve the crude curare as prepared by the Indians of South America and yet would not be as elaborate as the methods of King and others, would be of value. We have previously used liquid ammonia (3) as a solvent, dispersing agent and extractive for other biochemical compounds, and these methods have been extended to the study of curare. Preliminary tests showed that curare was not inactivated by contact with liquid ammonia for 48 hours. A progress report (4) of this work has been made.

METHODS. The anhydrous liquid ammonia (Mathieson) used in this work was chemically pure and did not require drying, and the curare (Merck) and the glycine (Pfanstiehl) were not subjected to any special drying. However, the reactions were carried out in a closed system of Dewar flasks and the excess ammonia gas was permitted to escape through a mercury seal in order to prevent the admittance of air and moisture. A considerable amount of moisture will condense upon the walls of an open flask containing liquid ammonia since the latter liquid boils at a temperature of -33.4°C . The filtrations were made through sintered glass filters enclosed in the Dewar flasks. The closing of the outlet on the reaction flask and the opening of the outlet on the receiving flask ordinarily created enough pressure to force the extractive liquid through the sintered glass filter, which was contained in the reaction flask, and to allow it to flow into the receiving flask. If more pressure was needed to hasten the filtration ammonia gas from the large ammonia cylinder could be admitted to the reaction flask. Excessive pressures within the glass system were not used since they are dangerous and otherwise undesirable for filtrations.

The bioassays of the various derivatives were made upon frogs, and the injections were made into the ventral lymph sac. A unit of paralyzing power for purposes of comparison was taken as that weight of ammonia-treated curare of a derivative that will paralyze in 15 minutes the righting reflex of a 10 gm. frog so that the animal cannot recover its normal position within one minute. The aqueous solutions or dispersions were made up to a strength of 2% in terms of curare which had first been placed in liquid ammonia and the latter then permitted to boil away; therefore, 0.05 cc. would contain 1.0 mgm. of the treated curare which was the amount found necessary to paralyze a 10 gm. frog under the conditions stated. Therefore, 1 mgm. of treated curare equalled 1 unit of paralyzing power for the batch of curare which was used in all of these experiments. Thus three solutions, A, B and C, wherein 0.05 cc. of A would paralyze a 10 gm. frog or 0.1 cc. of B would paralyze a 20 gm. frog or 0.15 cc. of C would paralyze a 30 gm. frog would all be considered of unit

strength. However, if 0.05 cc. of a fourth solution D would paralyze a 20 gm. frog, then solution D would be considered of double strength. Since medium sized frogs of about 20 gm. weight were used, an injection of 0.1 cc. of a new derivative would quickly show whether or not it was relatively stronger or weaker than the curare controls. Other comparisons such as milligrams treated curare per kilogram frog can be used, and in cases A, B and C the ratio is 100. For D the ratio would be 50. These are average values for several dozens of frogs and for the particular batch of curare used. Not less than 6 frogs were used for any assay. It should be remembered, of course, that each batch of curare must be standardized or assayed separately. The present batch of ammonia-treated curare then assays 1.0 mgm. = 1.0 unit of paralyzing power or a mgm./kgm. ratio of 100. The curare itself has a ratio greater than 100 as will be shown in the following pages.

RESULTS. When 100 mgm. of curare are added to 5 cc. of distilled water, or a concentration of 2%, it forms a greenish-brown suspension that is fine enough for injection, but which settles out on standing. However, if the curare has first been treated with liquid ammonia, the ammonia allowed to boil off and any remaining ammonia gas removed by a vacuum pump, it will either go into solution in water or form a very fine dispersion, which is stable for several hours. This

TABLE 1

CURARE DERIVATIVES AND UNTREATED CURARE CONTROL	ACTIVITY RATIO MGM. OF DRUG/KGM. OF FROG
1. Curare placed in liquid ammonia for 24 hours	100
2. Curare untreated (control)	150
3. Curare plus glycine placed in liquid ammonia for 24 hours	90
4. Curare plus glycine, soluble fraction passing 3G filter.	90
5. Curare plus glycine, insoluble fraction (less than 5%).	800
6. Curare, soluble fraction passing 3G filter	900
7. Curare, insoluble fraction not passing 3G filter	50
8. Curare, insoluble fraction not passing 3G filter, plus glycine	25

ammonia-treated curare colors the water a burgundy red. The greater solubility of the treated material is apparently accompanied by a better absorption which is equivalent to an increase in potency of 30%. This can be shown better by the ratios. The ammonia-treated curare has a ratio of 100, since on the average, a 20 gm. frog was paralyzed by 2 mgm. of treated curare and $\frac{2}{0.02} = 100$. For the curare controls 0.15 cc. of 2% curare (3 mgm.) was required to paralyze a 20 gm. frog and $\frac{3}{0.02} = 150$. Or 2 mgm. of ammonia-treated curare had the same effect as 3 mgm. of untreated curare in paralyzing a 20 gm. frog.

Since curare is somewhat soluble in liquid ammonia, giving to it an amber color, it seemed advisable to fractionate it and to test the potency of the soluble and insoluble fractions. This was done, using a Jena 3-G sintered glass filter as described above. It was found that four 100 cc. extractions on a 0.5 gm. sample removed 63.5% of it, and that the fraction of curare soluble in the liquid ammonia formed a black and shiny plate on the wall of the receiving Dewar flask, while the

insoluble fraction remained as an amorphous, very porous, tan-colored powder in the Dewar reaction flask. The activity of the two fractions varied considerably, most of the activity remaining in the fraction which was insoluble in liquid ammonia but soluble in water. An injection of 0.9 cc. of the fraction which was soluble in both liquid ammonia and water was required to paralyze a 20 gm. frog within 15 minutes. This corresponds to a mgm./kgm. ratio of 900. The insoluble fraction has a mgm./kgm. ratio of 50.

When an equal weight of glycine is added to the curare, and a fractionation is carried out as described above, 95 per cent of all of the material in the reaction flask is removed by one extraction only. This material has a mgm./kgm. ratio of 90, which ratio can also be obtained by simply adding glycine to curare in liquid ammonia and no filters used at all. The small amount of insoluble material left had a high ratio of 800. The glycine has a remarkable effect in making the active alkaloids of curare soluble in liquid ammonia. (See table 1.)

In view of the above facts it was decided to extract the curare with liquid ammonia first, thus removing more than 50% of inert, tar-like material contained in it, and then to treat the insoluble, but highly active, tan-colored powder with glycine. This was done, and an extremely potent and water soluble derivative was obtained. The mgm./kgm. ratio of this material was 25. It was six times as potent, mgm. for mgm., as the curare control used in this work. Furthermore, it seems to have a more prolonged paralyzing power and to be less toxic than curare. Ordinarily a frog which receives enough curare to prevent the righting reflex after 15 minutes will remain paralyzed for 18 to 48 hours and about 10% of them will die. However, the conjugate just described will, under the same conditions, paralyze a frog for 48 to 120 hours with less than half as many frogs failing to recover.

SUMMARY

1. Liquid ammonia does not inactivate curare by remaining in contact with it for 48 hours.
2. Curare which has been treated with liquid ammonia forms a much finer dispersion in water than does untreated curare.
3. Curare can be fractionated in liquid ammonia by the use of sintered glass filters with the removal of more than 50% of inert material.
4. The insoluble fraction of curare in liquid ammonia when treated with glycine becomes more potent, prolonged in action and less toxic than curare.

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A MIXED COLOR DITHIZONE METHOD FOR THE DETERMINATION OF BISMUTH IN BIOLOGICAL MATERIAL¹

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The defects in the commonly employed methods for the microdetermination of bismuth in biological material led us to undertake the development of a more adequate analytical technique for clinical and experimental studies, the primary objective being to devise a method which is more specific, more sensitive, and less laborious. The method proposed by Leonard (1), and subsequently modified by Hanzlik (2), has been most generally employed. However, we observed that this technique will yield high results when iron is present in quantities usually found in blood and other tissues. An appreciable error is introduced when one deals with the small quantities of bismuth present in blood even after bismuth therapy. The photometric dithizone method proposed by Hubbard (3) is specific and sensitive, but is too time-consuming for routine analyses because of the repeated transfers of the specimen and the necessity of preparing standard dithizone solutions.

The proposed method is an adaptation of the technique for the determination of lead reported by Kozelka and Kluchesky (4, 5). Bismuth can be determined quantitatively with a single extraction in the presence of interfering metals. The method is sensitive to one microgram and has proved reliable for all ranges of concentrations likely to be encountered in biological material. The greatest difficulty encountered in the determination of lead or bismuth with dithizone is the quantitative separation of these two metals. Willoughby, Wilkins and Kraemer (6) suggested adjusting the solution containing the lead and bismuth to pH 2 and extracting the bismuth with an excess of dithizone. Gant (7) observed that bismuth is extracted with difficulty at pH 2 but that very little remains in the aqueous phase at this pH when present as a dithizonate. He suggested separating the lead from the mixture of lead and bismuth dithizonates with a nitric acid solution of pH 2. These observations were confirmed in this laboratory. It was found that bismuth in the presence of the salts in biological digests is extracted with difficulty at pH 2 to 2.5, but that a quantitative extraction can be effected from a nitric acid solution in this pH range. A quantitative separation of the bismuth from the lead can be obtained by stripping the lead from the mixture of lead and bismuth dithizonates with a nitric acid solution of pH 2.3 to 2.5.

Reagents and apparatus. Sulfuric acid, concentrated. Nitric acid, concentrated. Hydrochloric acid, concentrated. Perchloric acid, 60 per cent. Sulfur dioxide, obtained in

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imeter are given in table 1. The values for the eleven points on the ordinate axis for the 5200 Å. readings, with the corresponding 6600 Å. values, are given for zero and thirty micrograms of bismuth. After the curves have been drawn through the points with the same 6600 Å. values, the spaces between these curves

TABLE 1

Logarithm values of galvanometer readings with the Evelyn photoelectric colorimeter for zero and 30 micrograms of bismuth (with increasing quantities of dithizone)

	MICROGRAMS OF BISMUTH		Log ₁₀ OF 6600 Å. FILTER READINGS*
	0	30	
Log ₁₀ of 5200 Å. filter readings with increasing amounts of dithizone	1.943	1.370	1.90
	1.920	1.340	1.85
	1.896	1.310	1.80
	1.872	1.282	1.75
	1.845	1.252	1.70
	1.818	1.220	1.65
	1.788	1.188	1.60
	1.756	1.152	1.55
	1.720	1.112	1.50
	1.682	1.071	1.45
	1.644	1.030	1.40

* Each logarithm of 6600 Å. reading corresponds to logarithms of 5200 Å. readings for the various quantities of bismuth.

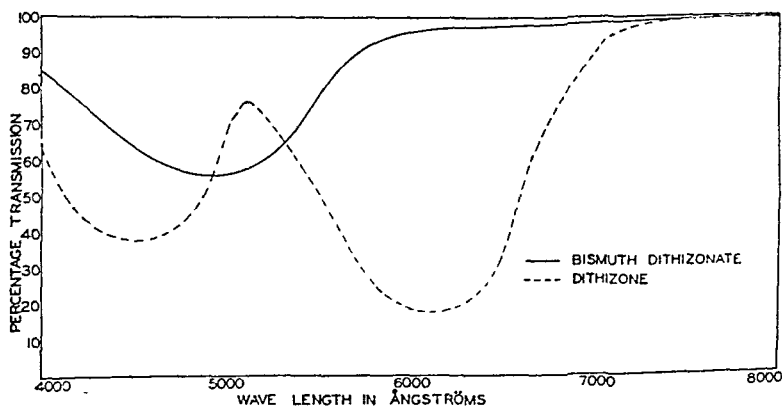


FIG. 2. TRANSMISSION OF LIGHT THROUGH DITHIZONE AND BISMUTH DITHIZONATE AT THE VARIOUS WAVE LENGTHS

are subdivided into five equal parts. Each subdivision represents a 0.01 variation in the 6600 Å. value. It will be noted in figure 2 that the maximum absorption of light by the bismuth dithizonate occurs in the region of 5000 Å. The filter employed is centered at 5200 Å. but covers a range from 4950 Å. to 5500 Å. and therefore serves the purpose adequately.

TABLE 2

Recoveries of known amounts of bismuth (from solutions containing known amounts of lead)

LEAD ADDED	BISMUTH ADDED	BISMUTH FOUND	DEVIATION
micrograms	micrograms	micrograms	micrograms
20	0	0.5	+0.5
20	0	0.3	+0.3
20	0	0.2	+0.2
20	10	9.6	-0.4
20	10	9.8	-0.2
20	10	9.9	-0.1
15	10	9.3	-0.7
15	10	9.3	-0.7
15	10	9.3	-0.7

TABLE 3

Recoveries of known amounts of bismuth from 10 grams of normal blood

LEAD PRESENT	BISMUTH ADDED	BISMUTH RECOVERED	DEVIATION
micrograms	micrograms	micrograms	micrograms
14	0	0.5	+0.5
14	5	4.8	-0.2
14	2	2.4	+0.4
14	8	7.9	-0.1
14	10	10.3	+0.3
14	12	11.6	-0.4
14	15	15.0	0.0
14	20	19.6	-0.4
14	22	23.2	+1.2
14	25	26.1	+1.1

TABLE 4

Recoveries of bismuth from 10 cubic centimeters of urine

LEAD PRESENT	BISMUTH ADDED	BISMUTH RECOVERED	DEVIATION
micrograms	micrograms	micrograms	micrograms
5	0	0.4	+0.4
5	0	0.4	+0.4
5	5	5.7	+0.7
5	5	4.5	-0.5
5	10	10.4	+0.4
5	15	15.5	+0.5
5	20	20.1	+0.1

The accuracy of the nomogram can be tested by extracting known quantities of bismuth from a copper-free dilute nitric acid solution, pH 2.3 to 2.5. For other types of photoelectric colorimeters the technique for obtaining the neces-

sary data for the construction of the nomogram is described by Kozelka and Kluchesky (4).

Results. Representative recoveries of bismuth in the presence of lead from pure solutions, blood, and urine are given in tables 2, 3 and 4. It will be noted that recoveries are obtainable with a mean error of less than one microgram. For quantities of bismuth larger than 25 micrograms, a smaller aliquot of the digest must be taken or a greater dilution of the bismuth dithizonate extract should be employed.

SUMMARY

A simplified mixed color dithizone method for the determination of bismuth in biological material is described. Treatment of the digest with sulfur dioxide prevents decomposition of the dithizone by the iron and eliminates tin as an interfering metal, hence permits the determination of bismuth with a single extraction. The method eliminates the necessity of removing the excess dithizone or preparing standard dithizone solutions. Consistent recoveries with a mean error of less than one microgram are obtainable.

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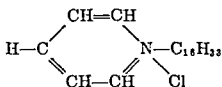
PHARMACOLOGICAL AND TOXICOLOGICAL STUDIES ON CETYLPYRIDINIUM CHLORIDE,* A NEW GERMICIDE

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Jacobs and his coworkers (1, 2, 3) in 1916 reported results of a systematic study of synthetic germicides in which they pointed out that quaternary ammonium salts possessed germicidal properties. In view of their therapeutic possibilities, a large series of compounds has been synthesized and tested in this laboratory. The results of these studies have been reported elsewhere (4, 5, 6). One of these compounds, cetylpyridinium chloride, gave indications of possessing very good germicidal properties and therefore was studied in more detail.

This compound has the chemical composition $C_{21}H_{35}NCl$ and the following structural formula:



It is a pure organic compound which occurs as a monohydrate, a white crystalline solid melting at 80°C . Cetylpyridinium chloride dissolves readily in water (1:5) forming water-clear, colorless, and almost odorless solutions. It is easily soluble in alcohols, sparingly soluble in fatty oils, and insoluble in ether.

The germicidal action of cetylpyridinium chloride, as well as the effect of the pH and the cetyl radical upon this action, has been reported previously (4, 5, 6, 7, 8, 9, 10). Using the standard Food and Drug Administration technique, it was found that in the absence of serum this compound killed *Staphylococcus aureus* in a dilution of 1:65,000 and in the presence of 10 percent serum it killed the same organism in a 1:11,500 dilution. Marked germicidal potency has also been demonstrated against other organisms. Detailed reports of these studies will be presented elsewhere.

In view of the possible clinical use of cetylpyridinium chloride as a germicidal agent and the fact that only a preliminary study (11) has been reported, it seemed desirable to record the results of a more thorough pharmacological and toxicological investigation.

EXPERIMENTAL STUDIES. *Toxicity.* The toxicity studies were of two general types, acute and chronic. In the acute tests the toxicity of cetylpyridinium chloride was studied following the administration of large doses by either the intravenous, intraperitoneal, subcutaneous, or oral route. These tests were carried out on mice, rats, guinea pigs, rabbits, and dogs. Since the toxic effect was delayed in many cases, the animals were observed for a period of a week to

* Cetylpyridinium Chloride ("Ceepryn" brand) produced by The Wm. S. Merrell Company.

ten days. Rabbits were used in most instances for the acute toxicity studies following intravenous administration. A 2.5 percent solution of the compound was employed and, since the rate of injection affects the results, a constant rate of 25 mgm. per minute was adopted. The results of these studies are summarized in table 1.

Lethal doses caused either convulsions or depression prior to death. When depression was absent, convulsions appeared at the completion of or soon after injection and these terminated in death. In some instances the period of convulsion was followed by depression and the animals died in this state. Some of the rabbits did not exhibit convulsions but became depressed immediately following injection and died.

The administration of maximum tolerated doses of cetylpyridinium chloride, 15-20 mgm. per kgm., produced either convulsions or a depression which was accompanied by paralysis of voluntary motion. At times both conditions were evident. If the animal survived for 30 or 35 minutes following the injection of large doses it usually recovered. Death was caused by asphyxia apparently due to paralysis of the respiratory muscles.

TABLE 1

Acute toxicity of cetylpyridinium chloride when injected intravenously in rabbits

	DOSE: MG./KGM.						
	15	20	25	30	35	40	45
No. of animals dead.....	0	1	4	4	6	7	6
No. of animals injected.....	12	12	12	12	12	12	6

On the basis of the results obtained it is apparent that the MLD of cetylpyridinium chloride in these tests is 20 mgm. per kgm. body weight and the LD₅₀ 35 mgm. per kgm.

Further studies were made on dogs. A male, weighing 4.85 kgm. received a dose of 25 mgm. per kgm. of cetylpyridinium chloride intravenously. Several minutes after the injection the muscles of the extremities became partially paralyzed. Approximately fifteen minutes later the paralysis subsided and the animal appeared normal.

Three days later another test was made on the same animal. Cetylpyridinium chloride in a dose of 30 mgm. per kgm. was administered intravenously. Again there was a paralysis of the extremities but the dog recovered rapidly. Thirty minutes after the first injection 40 mgm. per kgm. were administered. The animal became markedly depressed and exhibited convulsive movements five minutes after the completion of the injection. Eight minutes after the injection the convulsive movements had ceased and the animal attempted to arise but the posterior extremities were still paralyzed. Ten minutes after the injection the dog was up and soon appeared normal. Within a period of forty-five minutes this animal had received a total of 70 mgm. per kgm. of cetylpyridinium chloride. There were no delayed effects and a month later the animal was still normal and gaining weight.

To determine the type of death a female weighing 4.32 kgm. received a large amount of cetylpyridinium chloride, 100 mgm. per kgm., intravenously. Within a few minutes the muscles of all the limbs were paralyzed and the animal sank to the floor. This was soon followed by paralysis of the respiratory muscles and death.

Studies on the intraperitoneal toxicity of cetylpyridinium chloride were carried out on rabbits, guinea pigs, rats, and mice. A 2.5 per cent solution of the compound was injected intraperitoneally and the animals were observed for a period of one to two weeks. The results of these tests are summarized in table 2.

It will be noted that the toxicity of cetylpyridinium chloride following intraperitoneal injection was greater than that following intravenous administration. A further point of interest was its delayed action when administered by this route. The animal often did not exhibit any toxic symptoms for from 6 hours to

TABLE 2
Intraperitoneal toxicity of cetylpyridinium chloride

TEST ANIMAL	DOSE: MG./KG.						
	3	5	10	15	20	25	30
Rabbit:							
No. of animals dead.	0	3	4	3	4	8	
No. of animals injected	12	12	12	12	12	12	
Guinea pig:							
No. of animals dead.	0	3	5	9	11		11
No. of animals injected	12	12	12	12	12		12
Rat:							
No. of animals dead		0	0	4	3	7	9
No. of animals injected		12	12	12	12	12	12
Mouse:							
No. of animals dead	2	3	9	9	11		
No. of animals injected	12	12	12	12	12		

3 or 4 days. After this time some of the animals suddenly became depressed and died. Autopsy did not reveal any gross abnormalities; there was no inflammation at the site of injection.

The toxicity of cetylpyridinium chloride was further studied following subcutaneous injection. A 2.5 per cent solution of the compound was again employed and rabbits served as the test animals. Observations were made daily for a period of a week following administration. A summary of the results is presented in table 3. The toxic symptoms were delayed and similar to those observed following intraperitoneal administration. Death occurred 24 hours to 6 days following injection of a fatal dose. Amounts used in these tests produced a marked dehydration of the tissue followed by necrosis at the site of injection.

Rabbits served as test animals for the acute oral toxicity studies. A 10 per cent aqueous solution of cetylpyridinium chloride was used and the animals were again observed over a period of seven days. The results are summarized in table 4.

Most of the rabbits developed a diarrhea as a result of the administration of cetylpyridinium chloride in these large amounts. In lethal doses death occurred at intervals of one to 6 days. It is apparent that cetylpyridinium chloride was much less toxic by the oral route than by any of the other modes of administration.

Experiments were undertaken to study the effect of long continued administration of cetylpyridinium chloride in rabbits. For these tests the animals were divided into the following three groups: controls, those receiving 10 mgm. per kgm. orally per day, and those receiving 100 mgm. per kgm. orally per day.

All the dosages were calculated on the weight of the individual animal at the beginning of the experiment. The results of these tests are based on 6 controls and 22 treated animals, 12 of which received a daily dose of 10 mgm. per kgm. body weight of cetylpyridinium chloride while the remaining 10 received a daily dose of 100 mgm. per kgm.

TABLE 3
Subcutaneous toxicity of cetylpyridinium chloride in rabbits

	DOSE: MG./KGM.				
	100	150	200	250	300
No. of animals dead.....	0	0	2	2	3
No. of animals injected.....	6	6	6	6	6

TABLE 4
Acute oral toxicity of cetylpyridinium chloride in rabbits

DOSE: MG./KGM.	300	400	500	600	700
No. of animals dead.....	0	1	5	4	4
No. of animals injected.....	6	6	6	6	6

The majority of the animals gained weight during the test period. In every case where there was a loss in weight this was attributable to a temporary diarrhea. At the end of the four-week period one-half of the animals in the various groups were sacrificed and examined for any indications of gross pathological changes. Tissue was taken for histological examination. The remainder of the animals were allowed to live for another two-week period and then killed and examined. There was no gross pathological condition which could be attributed to the cetylpyridinium chloride.

None of the tissues with the exception of the liver and kidney presented any pathological changes. Many of the liver sections showed varying degrees of vacuolization of the cytoplasm of the hepatic cells. This rarefaction of the cytoplasm was more or less diffuse and not limited to any particular portion or zone of the lobule. Neither was it limited to the test animals but was also present in two of the control animals. It is questionable whether this cytoplasmic rarefaction can be considered as the true toxic effect of the compound since it appeared in all groups.

The kidney sections, like the liver sections, showed a vacuolization of the cytoplasm of the cells lining the tubules. This vacuolization was present in varying degrees although very slight in most cases. The rarefaction of the cytoplasm was apparent to some extent in all of the groups but was more pronounced in animals receiving the larger dose of the compound. There was also some cloudy swelling of the cells of the tubules, but no degeneration of the nuclei and no pathological changes in the glomeruli were seen.

The results of these tests indicate that the daily administration of cetylpyridinium chloride in these doses for a period of four weeks did not have any significant harmful or toxic effects.

Irritation. A number of different types of tests were made to determine the irritant properties of cetylpyridinium chloride. Included among these were studies on the effects of varying concentrations of the compound on scarified skin, conjunctival mucosa, the mucosa of the urinary bladder, healing, and following intracutaneous and subcutaneous injection.

Rabbits were used to determine the effect of local applications to the skin. Pads of gauze saturated with varying dilutions of cetylpyridinium chloride were applied to the scarified skin and held in place for a period of 24 hours. A concentration of 1:100 or 1:250 produced some reddening of the skin while greater dilutions had no effect.

The effect of intracutaneous injections was studied by injecting small amounts of varying concentrations of the compound. The amount administered in each case was 0.1 cc., sufficient to produce a small wheal. A 1:1000 isotonic solution produced a small area of tissue dehydration but there was no sloughing. Higher dilutions had no effect.

In further studies solutions of cetylpyridinium chloride were injected subcutaneously into rabbits in a volume of 1 cc. in each case. The concentrations used varied in strength from 1:100 to 1:1000. Two types of solutions were used: In one the cetylpyridinium chloride was dissolved in distilled water, and in the other the compound was made up as an isotonic solution with sodium phosphate. The unbuffered solution in a 1:100 concentration caused an area of necrosis at the site of injection and some edema when used in a 1:250 concentration. There was no effect with higher dilutions. The buffered solution in a 1:100 concentration produced a slight edema and reddening at the site of injection which disappeared after several days. Higher dilutions produced no untoward effect.

The effect of cetylpyridinium chloride on the conjunctival mucosa was studied in rabbits. The mucosa was irrigated with varying concentrations of the compound for a period of two minutes. The surplus solution was allowed to remain in contact with the mucosa. Unbuffered as well as isotonic solutions were employed. In general, the isotonic solution appeared to be slightly less irritating. A concentration of 1:1000 to 1:3000 of either solution produced a definite irritation of the conjunctival mucosa. If the solution was diluted to 1:5000 there was either no or, in some cases, a very slight indication of irritation.

A number of tests were made to study the effect of cetylpyridinium chloride on the mucosa of the urinary bladder of rabbits. The procedure was as follows:

10 cc. of an isotonic solution of cetylpyridinium chloride in varying concentrations were injected into the bladder by means of a catheter. The solution was allowed to remain in the bladder until voided. At various intervals the animals were killed and the bladder examined. A concentration of 1:100 produced marked inflammation, edema, and congestion of the bladder and external genitalia. This inflammation was still evident three or four days after the irrigation. A concentration of 1:500 produced a slight inflammation of the bladder wall in some animals but had no effect on others. When cetylpyridinium chloride was injected in a concentration of 1:1000 there was no indication of irritation or inflammation in any of the animals.

Since cetylpyridinium chloride produced marked irritation and sloughing in concentrated solutions it was of interest to determine whether it would interfere with the healing processes. Rabbits were anesthetized and two incisions made under aseptic conditions in the lumbar musculature of each animal. In every case one of the incisions was irrigated with a 1:1000 solution of cetylpyridinium chloride and the other served as a control. The sides of the incisions were approximated by means of several sutures and the animals were observed daily to note the rate of healing. There seemed to be no difference. Animals were killed at varying intervals and a section of the tissue from the site of the incision was removed and prepared for microscopic study. The results indicate that this compound did not interfere with the healing processes under these conditions.

Effect on the respiratory and cardiovascular systems. A series of preliminary tests were made to determine the effect of cetylpyridinium chloride on respiration and on the cardiovascular system following intravenous administration. Dogs were used in these studies and they were anesthetized with sodium pentobarbital, 30 mgm. per kgm., administered intravenously. Cetylpyridinium chloride in doses of 1 or 5 mgm. per kgm. had very little effect on the respiration. In a dose of 1 mgm. per kgm. there was a marked bradycardia of central origin since it was prevented by atropine. If cetylpyridinium chloride is administered in a dose of 5 mgm. per kgm. there is an initial marked increase in the pulse rate followed by a more gradual decrease to below normal.

A series of preliminary tests were made to determine the effect of cetylpyridinium chloride on the blood pressure of dogs. Regardless of the type of anesthesia or of the dose the blood pressure curve was more or less typical. There was an initial temporary fall followed by a rise above the normal level and then a secondary drop below normal. Since the primary drop was usually present in atropinized dogs and since pithing did not prevent it, it does not appear to be of central origin. Nicotization of the dogs, furthermore, did not prevent the secondary rise.

Discussion. Crum, Brown and Fraser (12) in 1869 reported the "curar-like" action of some quaternary ammonium bases. This same type of action is also apparent in many of the more complex amines containing a nitrogen atom in the ring, e.g. pyridinium. That cetylpyridinium chloride manifests this "curar-like" action seems to be indicated from the results of a number of the tests.

dose, 15 to 20 mgm. per kgm., a paralysis occurred which spread to the abdominal and respiratory muscles. This same type of reaction was observed in dogs following an intravenous dose of 25 to 30 mgm. per kgm.

When cetylpyridinium chloride was administered intraperitoneally to rats in a dose of 5 mgm. per kgm. the animals began to exhibit a paralysis in about 12 minutes. The animals at this time attempted to walk but rolled over on their side or back; however, in a few minutes they arose. This procedure was repeated at irregular intervals. The posterior limbs were more affected than the anterior ones; the paralysis seemed to affect the feet first and then to progress upward. Regardless of the mode of administration or the test animal, paralysis occurred if a sufficient amount of the compound was administered.

A central stimulating action of cetylpyridinium chloride was apparent from results of some tests on frogs. It is well known that the application of curare directly to the spinal cord of frogs produces typical strychnine convulsions. If a small amount, 0.01 cc. of a 1:100 solution, of cetylpyridinium chloride is injected intracranially in frogs at the juncture of the cord and brain stem, typical strychnine convulsions result. In control tests similar amounts of a saline solution may be injected without any apparent effect. The results of these tests indicate that cetylpyridinium chloride produces a central stimulation as well as a peripheral paralysis.

The increased toxicity as well as the delayed action of cetylpyridinium chloride following intraperitoneal administration is of particular interest. At present no logical explanation can be advanced for these results.

Cetylpyridinium chloride exerts a definite action on the cardiovascular system. This effect is very much the same regardless of whether the compound is administered to an animal anesthetized with sodium phenobarbital or pithed. More studies are necessary before this action can be explained.

SUMMARY

The acute as well as the chronic toxicity of cetylpyridinium chloride has been determined. The MLD in rabbits following intravenous administration was 20 mgm. per kgm. Following oral administration this compound in an amount of 400 mgm. per kgm. killed one out of six rabbits. It was found that cetylpyridinium chloride was most toxic following intraperitoneal administration. In many cases death was delayed for from three to six days.

The results of the chronic toxicity tests indicate that cetylpyridinium chloride in the amounts administered for a period of four weeks has no significant harmful effects.

The irritant action on intact skin and mucous membranes was determined.

Cetylpyridinium chloride exerted a "curare-like" action. It produced a central stimulation and a peripheral paralysis.

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